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## ERRATA

The Journal of Nutrition, vol. 38, no. 1, May, 1949.

A. Z. HOBSON

Page 27, table 1, under "Fresh Milk 3" and under "Evaporated Milk 3": the word *and* should be inserted after "acids" so the two passages will read "Proteins precipitated with hydrochloric and metaphosphoric acids and at pH 7.0."

Pages 32 and 33, tables 4 and 5: the numerical values should read  $\mu\text{g/l}$  instead of  $\mu\text{g/gm}$ .



# THE ENERGY EXPENDITURE OF BOYS AND GIRLS 9 TO 11 YEARS OF AGE (1) SITTING LISTENING TO THE RADIO (PHONOGRAPH), (2) SITTING SINGING, AND (3) STANDING SINGING

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in cooperation with the Bureau of Human Nutrition and Home Economics,  
U. S. Department of Agriculture, Washington, D. C.*

ONE FIGURE

(Received for publication January 7, 1919)

Among the many activities in which 9- to 11-year-old boys and girls engage, that of sitting listening to the radio has been found to be one of the favorite indoor pastimes. Children also sing a great deal both at home and at school. In the interest of increasing our knowledge of the energy requirements of children, and in view of the fact that the energy expenditure of children engaged in these activities had not been previously investigated, such studies were undertaken.

## EXPERIMENTAL

### *Subjects*

The children came from backgrounds similar to those of the children reported on in studies by Taylor, Pye and Caldwell ('48). Although a number of the same children participated in the study of each of the three activities, it was necessary to work with new children from time to time since some grew out of the age range and a good representation of children within the age range was desired. In all, 22 children





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TABLE 1

*Average weight, height, deviation from predicted weight, and basal metabolism of children studied (figures in parentheses show range)*

SUBJECTS	BODY WEIGHT	BODY HEIGHT	DEVI- ATION FROM PRE- DICTED WEIGHT <sup>1</sup>	BASAL METABOLISM IN CALORIES PER			
				24 hr.	kg/ hr.	m <sup>2</sup> / hr.	cm of ht./ 24 hr.
	kg	cm	%				
Boys, <sup>2</sup> Ages 9 yr. 4 mo. to 11 yr. 5 mo.							
Group I							
12 boys serving as subjects for listening to radio	37.6 (30.8- 46.2)	143.9 (136.5- 153.3)	+ 3.0 (- 7.9- + 14.5)	1287 (1186- 1543)	1.44 (1.21- 1.69)	43.7 (39.2- 48.8)	8.9 (8.3- 10.3)
Group II							
6 boys serving as subjects for sitting singing	36.8 (31.6- 44.1)	144.1 (136.5- 150.6)	+ 1.6 (- 7.9- + 12.0)	1306 (1186- 1426)	1.45 (1.35- 1.63)	44.6 (42.8- 48.8)	9.1 (8.5- 9.7)
Group III							
8 boys serving as subjects for standing singing	37.0 (31.6- 44.1)	144.1 (136.5- 151.0)	+ 0.9 (- 7.9- + 12.0)	1285 (1186- 1426)	1.49 (1.35- 1.63)	43.8 (40.5- 48.8)	8.9 (8.3- 9.7)
Girls, <sup>2</sup> Ages 8 yr. 9 mo. to 10 yr. 11 mo.							
Group I							
10 girls serving as subjects for listening to radio	33.4 (28.1- 41.1)	137.6 (126.8- 149.6)	+ 2.1 (- 8.3- + 14.3)	1191 (1114- 1331)	1.50 (1.34- 1.67)	43.9 (41.4- 48.3)	8.7 (8.1- 9.4)
Group II							
9 girls serving as subjects for sitting and standing singing	35.5 (29.3- 50.0)	140.6 (126.8- 156.0)	+ 2.4 (- 8.3- + 11.7)	1228 (1105- 1531)	1.46 (1.28- 1.67)	43.5 (41.4- 48.3)	8.7 (8.1- 9.8)

<sup>1</sup> Deviations from Baldwin-Wood standards.

<sup>2</sup> Many of the same boys — or girls — were subjects for each activity. In cases where they outgrew the age range or were unavailable before sufficient tests on a given activity were obtained, additional subjects were included.

(12 boys and 10 girls) served as experimental subjects for the studies of the energy expenditure of children sitting listening to the radio (phonograph), 15 children (6 boys and 9 girls) for sitting singing, and 17 children (8 boys and 9 girls) for standing singing. Health records reported by physicians were consulted to determine the general health status of the children before they were asked to serve as experimental subjects. The weight, height, and deviations from predicted weight for height and age (Baldwin-Wood standards) were determined. The average values are given in table 1. Although the children varied in national background, they were all born in this country.

### *Procedure*

Basal metabolism determinations were made on all subjects under standard conditions at the beginning of the experimental period by means of the Collins-Benedict-Roth respiration apparatus. Not less than two determinations on each of two mornings were made on each child. More determinations were made when necessary in order to obtain an average of three of the lowest results agreeing within 5%. The activity studies in each case were completed within a period of less than 6 months following the basal metabolism determination. Whenever an experimental subject was continued for a longer period than this, another basal metabolism determination was made. The children included in the study had basal metabolisms which agreed within the commonly accepted normal range of  $\pm 15\%$  with two or more of the standards currently used for children.

\*The open-circuit respiration chamber described by Taylor, Lamb, Robertson and MacLeod ('48) was used for the activity studies. Two boys or two girls were studied at a time. The total carbon dioxide produced was divided by two to obtain the average carbon dioxide production per child and heat production computed as described in the paper just cited. The Carpenter modification of the Haldane gas analysis ap-

paratus was used for determining the carbon dioxide content of chamber air.

The activity studies were made after school. Since it was not possible to use a radio in the respiration chamber, programs for children similar to those on the radio had to be especially planned for each test. A phonograph was used and suitable records were selected from the Teachers College library collection for 9- to 11-year-old children. The phonograph was operated by an observer outside of the chamber and the stories or songs were broadcast into the chamber by means of an army two-way speaking system such as was used for communication in tanks and planes during the war (fig. 1). A field phone was placed in the chamber at such an elevation as to provide clear reception for the children in a comfortable sitting position. The same equipment was used for the study of the energy expenditure of children sitting listening to the recordings, and sitting and standing singing.

All tests were preceded by a 10-minute rest period. During the rest period the children sat quietly reading or playing with puzzles, games, or engaging in some other activity involving only finger muscles. Sometimes the rest period was followed by a period of sitting listening to the phonograph and at other times by one of the singing periods.

The storytelling period was planned for 20 minutes but the actual time varied from 18 to 23 minutes, depending upon the length of the story record and whether the voice of the storyteller necessitated an adjustment of the speed to insure clarity. The children sat and listened attentively, often spontaneously making gestures in an attempt to dramatize parts of the story. The records had to be carefully timed for the proper speed and the afternoon program rehearsed by the observers in advance so that the experimental period would approximate 20 minutes. Care was taken to avoid stories which were too young for the older children in the group in order to eliminate the possibility of muscle tension due to boredom.



Fig. 1 Two young boys standing singing in the respiration chamber. The two-way system is shown on table in front of respiration chamber, along with phonograph. The field phone, mounted on standard, can be seen through chamber window.

The same care was necessary in the selection of the songs for the singing periods. The program for the singing periods also had to be carefully timed and rehearsed by the observers in advance for an experimental period approximating 10 minutes. The children were given typed copies of the words of the songs before they entered the respiration chamber, and the recorded voice was clear enough for the children to follow easily so that there was continuous singing during the experimental period. The two activities, (1) sitting singing and (2) standing singing, were never studied on the same afternoon because the children could not be depended upon to sing enthusiastically and continuously for longer than 10 minutes at a time.

#### RESULTS AND DISCUSSION

The average results of these studies expressed in terms of total Calories per hour per kilogram of body weight and per centimeter of height are given in table 2.

##### *(1) Listening to the phonograph*

The average energy expenditure of the boys when sitting listening to the phonograph was  $2.07 \pm 0.06$  Cal. per kilogram of body weight per hour and  $0.54 \pm 0.01$  Cal. per centimeter of height per hour, and of the girls  $1.80 \pm 0.04$  Cal. per kilogram of body weight per hour and  $0.45 \pm 0.01$  Cal. per centimeter of height per hour. It will be noted that the boys spent more energy than the girls when engaging in the same activity. This is in line with the results of other studies from this laboratory. The coefficients of variation are, respectively, 13.0% and 11.9% for the boys, and 10.5% and 12.8% for the girls. When the probable error is expressed as per cent of the mean it is small, being only 2.8% and 2.6% for the boys and 2.0% and 2.5% for the girls. It is the opinion of the authors, resulting from observations of children sitting listening to the radio under nonexperimental conditions, that the amount of activity in which the children engaged under the

TABLE 2

*Average energy expenditure of children 9 to 11 years old, for (1) listening to the radio (phonograph), (2) sitting singing, and (3) standing singing (including the basal metabolism)*

	LISTENING TO THE RADIO (PHONOGRAPH)		SITTING SINGING		STANDING SINGING	
	Boys	Girls	Boys	Girls	Boys	Girls
	(10 cases)	(12 cases)	(10 cases)	(12 cases)	(11 cases)	(11 cases)
<i>CO<sub>2</sub> (gm) produced per child per hr.</i>						
Mean $\pm$ P.E. <sup>1</sup>	26.06 $\pm$ 0.75	20.49 $\pm$ 0.64	27.05 $\pm$ 0.89	21.39 $\pm$ 0.82	28.78 $\pm$ 0.78	25.09 $\pm$ 0.56
C.V. <sup>2</sup> (%)	13.6	16.1	15.4	17.2	13.3	12.0
P.E. as % mean	2.9	3.1	3.3	3.4	2.7	2.2
<i>Cal. per kg per hr.</i>						
Mean $\pm$ P.E.	2.07 $\pm$ 0.06	1.80 $\pm$ 0.04	2.23 $\pm$ 0.06	2.06 $\pm$ 0.07	2.35 $\pm$ 0.05	2.13 $\pm$ 0.05
C.V. (%)	13.0	10.5	13.2	17.5	10.7	11.2
P.E. as % mean	2.8	2.0	2.8	3.4	2.2	2.5
<i>Cal. per cm ht. per hr.</i>						
Mean $\pm$ P.E.	0.54 $\pm$ 0.01	0.45 $\pm$ 0.01	0.57 $\pm$ 0.02	0.52 $\pm$ 0.02	0.60 $\pm$ 0.01	0.54 $\pm$ 0.01
C.V. (%)	11.9	12.8	14.0	15.9	11.6	11.2
P.E. as % mean	2.6	2.5	3.0	3.1	2.4	2.1

<sup>1</sup> Probable error.

<sup>2</sup> Coefficient of variation.



experimental conditions of this study closely approximated that of the nonexperimental children, even though some of the programs might well be judged less exciting than many radio programs for children

### *(2) Sitting singing*

The average energy expenditure of the boys for sitting singing was  $2.23 \pm 0.06$  Cal. per kilogram of body weight per hour and  $0.57 \pm 0.02$  Cal. per centimeter of height per hour, and of the girls  $2.06 \pm 0.07$  Cal. per kilogram of body weight per hour and  $0.52 \pm 0.02$  Cal. per centimeter of height per hour. The energy expenditure of the boys was slightly above that of the girls, and the energy expenditure for sitting singing of both boys and girls was above that of both for sitting listening to the radio, although the difference is very small in each case. Although the children were not always equally enthusiastic about singing, on the whole both boys and girls cooperated well. All precautions were taken to insure the interest of the children and to duplicate, as far as possible, actual situations.

Studies of the energy expenditure of boys of this age seated and engaged in quiet play have been reported by Taylor ('37), and of girls by Potgieter ('33) and Thompson ('40). Other studies of boys and girls of this age engaged in other activities where the children were seated have been reported by Söndén and Tigerstedt (1895), Olin ('16), and Bedale ('23). The findings of all of these investigators where the children were seated performing different tasks but with similar degrees of activity are of the same order of magnitude as those of this study and will be discussed in detail in a later paper from this laboratory.

### *(3) Standing singing*

The average energy expenditure of the boys standing singing was  $2.35 \pm 0.05$  Cal. per kilogram of body weight per hour and  $0.60 \pm 0.01$  Cal. per centimeter of height per hour, and

of the girls  $2.13 \pm 0.06$  Cal. per kilogram of body weight per hour and  $0.54 \pm 0.01$  Cal. per centimeter of height per hour. The coefficients of variation are of the same order of magnitude as for the other activities, with a slight tendency to be lower. When the probable error of the mean is expressed as per cent of the mean it again is small, as shown in table 2. The energy expenditure for standing singing for both boys and girls is only slightly above that for sitting singing. The children stood quietly for the most part with occasional swaying and sang with varying degrees of enthusiasm. A few of the children responded to some of the songs having certain rhythms by tapping their feet while singing. Potgieter's studies (33) of the energy expenditure of girls of this age range standing relaxed are in agreement with the results of this study.

#### SUMMARY

The energy expenditure of 22 children (12 boys and 10 girls), 9 to 11 years of age, was measured while they were sitting in a respiration chamber listening to the phonograph. The average energy expenditure of the boys was  $2.07 \pm 0.06$  Cal. per kilogram of body weight per hour (coefficient of variation, 13.0%) and  $0.54 \pm 0.01$  Cal. per centimeter of height per hour (coefficient of variation, 11.9%); and of the girls  $1.80 \pm 0.04$  Cal. per kilogram of body weight per hour (coefficient of variation, 10.5%) and  $0.45 \pm 0.01$  Cal. per centimeter of height per hour (coefficient of variation, 12.8%).

The energy expenditure of 15 children (6 boys and 9 girls) was measured while they were sitting singing. The average energy expenditure of the boys was  $2.23 \pm 0.06$  Cal. per kilogram of body weight per hour (coefficient of variation, 13.2%) and  $0.57 \pm 0.02$  Cal. per centimeter of height per hour (coefficient of variation, 14.0%); and of the girls  $2.06 \pm 0.07$  Cal. per kilogram of body weight per hour (coefficient of variation, 17.5%) and  $0.52 \pm 0.02$  Cal. per centimeter of height per hour (coefficient of variation, 15.9%).

The energy expenditure of 17 children (8 boys and 9 girls) was measured while they were standing singing. The average energy expenditure of the boys was  $2.35 \pm 0.05$  Cal. per kilogram of body weight per hour (coefficient of variation, 10.7%) and  $0.60 \pm 0.01$  Cal. per centimeter of height per hour (coefficient of variation, 11.6%); and of the girls  $2.13 \pm 0.06$  Cal. per kilogram of body weight per hour (coefficient of variation, 14.2%) and  $0.54 \pm 0.01$  Cal. per centimeter of height per hour (coefficient of variation, 11.2%).

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# PTEROYLGLUTAMIC ACID AND REPRODUCTION IN THE RAT<sup>1</sup>

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(Received for publication December 18, 1948.)

Nelson and Evans (37) have shown that on the day of breeding a young female rat may be fed a purified diet containing succinyl-sulfathiazole (SST) and supplemented with all the known vitamins with the exception of pteroylglutamic acid (PGA) and proceed normally with this gestation. When this PGA-deficient diet was fed one to three months prior to breeding, marked upsets in reproduction occurred, i.e., resorption, decreased number of young per litter, and increased percentage of young born dead. Rats three to 4 months of age were used in this study.

The present communication reports (1) the reproductive performance of rats in which the deficiency was instituted at an earlier age (one to two months), (2) the accentuating effects of a chemical antagonist of pteroylglutamic acid, and (3) the curative effects of synthetic PGA. Consideration of

<sup>1</sup> Aided by grants from the Roche Anniversary Foundation, the Board of Research and the Department of Agriculture of the University of California, and the Rockefeller Foundation, New York City. We are indebted to Dr. T. H. Jukes, Lederle Laboratories, Inc., Pearl River, New York, for generous supplies of synthetic pteroylglutamic acid and of the chemical antagonist for pteroylglutamic acid; to Dr. W. A. Feirer of Sharp and Dohme, Inc., Glenolden, Pennsylvania, for succinylsulfathiazole; to Dr. E. L. Sevringhaus of Hoffmann-LaRoche, Inc., Nutley, New Jersey, for crystalline *d*-biotin; and to Dr. Randolph Major of Merck and Company, Inc., Rahway, New Jersey, for crystalline B vitamins, alpha-tocopherol and 2-methyl-1, 4-naphthoquinone.

the possible role of diminished food intake was obviated by the institution of paired feeding controls.

#### EXPERIMENTAL

Normal female rats (Long-Evans strain) from one to three months of age were placed on the PGA-deficient diet and bred with normal males after one, two or three months of the deficiency. Additional groups of animals, three to 4 months of age, were bred with normal males and placed on the deficient or supplemented diet the day of mating. Vaginal smears were examined daily during gestation for the presence of erythrocytes, the sign that implantation has occurred; all rats were weighed at regular intervals. The animals were kept on screens until the day before littering was expected to occur. All rats were autopsied a few hours after parturition had taken place, except when weight loss indicated resorption had begun autopsies were performed as early as the 20th day of gestation. The uterus was carefully examined for the presence of implantation or resorption sites.

The PGA-deficient diet was the same as that used previously by the present authors (Nelson and Evans, '47). It consisted of 24% alcohol-extracted casein, 63% sucrose, 8% hydrogenated cottonseed oil,<sup>2</sup> 4% salts No. 4, and 1% succinylsulfathiazole (SST). Crystalline vitamins per kilogram of diet were: 300  $\mu$ g *d*-biotin, 5 mg 2-methyl-1, 4 naphthoquinone, 5 mg thiamine HCl, 5 mg pyridoxine HCl, 10 mg riboflavin, 10 mg *p*-aminobenzoic acid, 20 mg nicotinic acid, 50 mg *d*-calcium pantothenate, 400 mg inositol and 1.0 gm choline chloride. Control rats received the identical diet supplemented with 5.5 mg synthetic PGA per kilogram of diet. Each rat received weekly a fat-soluble vitamin mixture containing 800 U.S.P. units vitamin A, 115 Chick Units vitamin D, 6 mg synthetic alpha-tocopherol, and 650 mg corn oil.<sup>3</sup>

The chemical antagonist of PGA used was that described by Franklin et al. ('47a), who reported that this "crude" syn-

<sup>2</sup> Crisco or Primex.

<sup>3</sup> Mazola.

thetic preparation<sup>4</sup> nullified otherwise adequate amounts of pteroylglutamic acid in the case of *Streptococcus faecalis* R, *Lactobacillus casei*, and the rat. Franklin, Stokstad and Jukes ('47b) described the acceleration of PGA deficiency by this preparation in mice and in chicks. Additional observations of its effects on the chick have been made by Hertz ('48c) and on the pig by Welch et al. ('47).

### RESULTS

Table 1 summarizes the reproductive performances of rats of different ages after varying periods of PGA-deficiency. The data for additional rats, 4 months old, placed on the purified SST diets the day of breeding are given to show again that this procedure does not impair the reproductive mechanism. It will be noted that there is little interference with implantation, as only one deficient group (after two months of prior deficiency) shows a failure of implantation greater than 10%. The average failure of implantation for all groups with deficiency prior to breeding (85 litters) is 7%, the identical value for all groups placed on the experimental diets the day of breeding (42 litters).

Although the variability among groups is marked, the data show that one month of prior deficiency, regardless of the age of the rats, interferes slightly with reproductive performance; two months markedly; and three months less markedly. Eleven per cent (range 0-25) of the rats resorbed after one month on the deficient diet, 44% (range 29-56) after two months, and 22% (range 13-33) after three months of prior deficiency. This decreased incidence of resorption after three months suggests some adjustment to the diet made during this prolonged period. There is little correlation with age and incidence of resorptions. The youngest rats (30-35 days old at the beginning of the experimental period) had the highest percentage of resorptions after one and two months

<sup>4</sup> Lot no. 125 was used in these studies without further purification.

TABLE 1  
*Effect of pteroylglutamic acid (PGA) deficiency on reproduction*

TIME DIETS STARTED	RATS BRED	AGE WHEN BRED	BODY WT. WHEN BRED	FAILED IMPLAN- TATION	RESORPTIONS OF IMPLAN- TATIONS	LITTERS OF IMPLAN- TATIONS	TOTAL YOUNG	YOUNG BORN DEAD	AV. YOUNG PER LITTER	AV. WT. OF YOUNG
	no.	days	gm	%	%	%	no.	%	no.	gm
PGA-supplemented rats										
Day of breeding	12	129	217	17	0	100	81	0	8.1	5.5
Average	10	130	219	0	0	100	87	0	8.7	5.9
	22	129	218	9	0	100	168	0	8.4	5.7
PGA-deficient rats										
Day of breeding	12	128	218	8	0	100	101	4	9.2	5.2
Average	12	130	221	0	0	100	112	0	9.3	5.5
	24	129	220	4	0	100	213	2	9.3	5.4
34 days before breeding	9	65	178	10	25	75	35	11	5.0	4.3
Average	10	100	219	10	0	100	82	4	9.1	4.7
	10	127	230	0	10	90	64	9	7.1	4.7
	29	95	210	7	11	89	181	8	7.3	4.6
63 days before breeding	8	94	228	0	56	44	18	0	4.5	4.7
Average	10	130	224	30	29	71	36	6	7.1	5.5
	9	156	245	0	44	56	29	3	5.8	5.8
	27	128	232	11	44	56	83	3	6.0	5.4
94 days before breeding	8	124	222	0	13	87	45	4	6.4	5.4
Average	10	162	247	0	20	80	46	4	5.3	5.3
	10	181	260	10	33	67	32	6	5.3	5.5
	28	159	245	3	22	78	123	4	5.8	5.4

of deficiency but the lowest after three months. Resorptions, according to the beginning age of the animals, averaged 31% for those starting at one month, 16% for those two months old, and 29% for those three months old. It is possible that the intermediate group of rats (two months of age) may have had greater vitamin stores than the other groups for some reason.

The percentage of young born dead is consistently higher than normal (i.e., 8%, 3% and 4%) after one, two, or three months of prior deficiency. The average number of young per litter is slightly decreased after one month of deficiency (7.3 young) and significantly decreased after two or three months of prior deficiency (6.0 and 5.8 young, respectively). The average weight of the young at birth is significantly decreased only for the group with one month of prior deficiency.

### *Effect of undernutrition and PGA supplementation*

In order to evaluate the role of undernutrition and the curative effects of PGA supplementation on these reproductive upsets, paired feeding studies were carried out. The rats were placed on the PGA-deficient diet at weaning (21 days) and bred with normal males after 60 days of the deficiency. (In the preceding study two months of prior deficiency had resulted in the highest incidence of resorptions.) One-third of the animals were continued on the PGA-deficient diet and their food intake measured daily; one-third received the PGA-supplemented diet in the same amount as was consumed by the corresponding deficient rats on the same day of gestation; and the remaining one-third received the PGA-supplemented diet ad libitum. The rats were carefully paired<sup>2</sup> with respect to age and body weight on the day of breeding

<sup>2</sup> The PGA-deficient group averaged 85 days of age and 199 gm in body weight the day of breeding after 63 days of deficiency with an average weight gain of 153 gm. The corresponding averages for the control groups were as follows: pair-fed controls—85 days, 196 gm; 64 days, 150 gm; ad libitum controls—86 days, 196 gm; 65 days, 151 gm; ad libitum group receiving the PGA-supplemented diet throughout the entire experimental period—85 days, 199 gm; 64 days, 153 gm.



and with regard to length of deficiency period and weight gain during this time. A fourth group was selected from additional animals placed on the PGA-supplemented diet at weaning and likewise bred after 60 days on the experimental diet.

The experimental data are summarized in table 2. In the deficient group two rats (20%) resorbed while the remainder (80%) littered. This incidence of resorptions is considerably lower than that previously found after two months of deficiency and emphasizes the variability to be expected. The average weight of the deficient young at birth was significantly lower than normal but the number of young per litter and the percentage of young born dead were not adversely affected.

In the pair-fed group, as in all other PGA-supplemented groups, 100% of the rats littered, showing that limitation of food intake is not the cause of resorptions in the deficient group. The birth weight of the young from pair-fed mothers was significantly higher than that of deficient young but did not attain the normal birth weights of the ad libitum groups. The restriction in food intake varied from 52 to 92% (average 76%) of the food eaten by the rats given the PGA-supplemented diet ad libitum. The increased efficiency of food utilization by rats receiving the synthetic vitamins is only slight (no significant difference), as can be seen by comparing the weight changes during the 20-day gestation period of the deficient and pair-fed groups, i.e., 45 gm and 55 gm, respectively. This is to be expected, inasmuch as the deficiency produced by the experimental procedure was only slight and resulted in a low incidence of resorptions.

The high percentage of young born dead in the two control groups subjected to the deficiency for two months prior to breeding should be noted. Although these high values (18% for the pair-fed group and 13% for the ad libitum group) are due principally or entirely to one dead litter in each group, they suggest that the addition of the synthetic vitamin, at the

TABLE 2  
Effects of food restriction and of PGA supplementation on reproduction with 64 days of PGA deficiency prior to breeding

EXPERIMENTAL GROUP	NO. BREED	BODY WT. AT MATING	WT. CHANGE DURING GESTATION <sup>1</sup>	AV DAILY FOOD INTAKE <sup>2</sup>	PAILED FETUSES	RESORPTIONS OF FETUSES	EMBRYONIC DEATHS	POSTNATAL DEATHS	POSTNATAL SURVIVORS	WEANING WEIGHT <sup>3</sup>
PGA-deficient	10	199	+45 ± 7.2 <sup>4</sup>	11.5 ± 0.6	0	0	0	0	0	1.0
PGA-supplemented pair-fed controls	10	196	+55 ± 5.5	11.5 ± 0.6	0	0	0	0	0	1.0
PGA-supplemented ad libitum controls	10	196	+95 ± 6.9	15.2 ± 0.6	0	0	0	0	0	1.0
PGA-supplemented controls <sup>1</sup>	10	199	+89 ± 2.7	14.5 ± 0.4	0	0	0	0	0	1.0

<sup>1</sup> The first three groups received the PGA-deficient diet 64 days prior to breeding and the two groups mated 20 days prior to breeding received the PGA-supplemented diet throughout the entire experimental period and demonstrated the prophylactic effects of the vitamin.

<sup>2</sup> Data determined during the 20 days following mating.

<sup>3</sup> Data determined during 21-22 days following mating.

<sup>4</sup> Standard deviation of the mean.

level used, only during gestation was not sufficient to counteract all the effects of the prior deficiency period. There was complete normality of the young from the control group receiving the vitamin prior to breeding.

### *Effect of the antagonist*

To accentuate the deficiency during reproduction, 5 gm of the "crude" antagonist per kilogram (i.e., 0.5%)<sup>6</sup> were added to the PGA-deficient diet. Normal females, three months of age, were placed on the deficient diet 10 to 15 days prior to breeding or on the day of breeding. Control rats received the identical diet supplemented with 50.5 mg synthetic PGA per kilogram diet, i.e., almost 10 times the usual level of 5.5 mg per kilogram diet.

Table 3 shows that the addition of 0.5% of the antagonist to the PGA-deficient diet results in 100% resorptions either with or without a prior deficiency period. PGA supplementation during gestation was not sufficient to counteract completely the effects of a prior deficiency period, as shown by the occurrence of resorption in this group; in addition, the number of young per litter was decreased slightly. When there was no prior deficiency period, PGA supplementation resulted in 100% litters with a normal number of young per litter. The marked differences in body weight change during gestation for the PGA-deficient and supplemented groups should be noted, as well as the fact that the deficient rats resorbed despite the gain in weight during the gestation period.

<sup>6</sup> Preliminary studies had shown that the addition of 1 gm of the antagonist per kilogram diet had little effect on reproduction. The addition of 10 gm antagonist per kilogram diet resulted in an acute PGA deficiency and 100% of the rats placed upon such a diet the day of breeding resorbed instead of littering. This acute deficiency in adult rats was characterized by an early loss of the estrous cycle in 15-30 days, a marked weight loss after 20 days, and death in 30-50 days. A marked anemia and leukopenia (especially granulocytopenia), severe oral lesions and deposition of pigment were noted in agreement with the observations of Franklin et al. ('47a) on weanling rats receiving similar levels of the antagonist.

TABLE 3  
Effect of the antagonist on PGA deficiency during reproduction

TIME DICES STARTED	RAT <sup>1</sup>	BODY WT. WHEN BRED	WT. CHANGE DURING GESTATION <sup>1</sup>	FAILED IMPLAN. TATION	REPRODUCTION <sup>1</sup> OF IMPLAN. TATION	DIFFEREN- CE BETWEEN TATION	NO. OF SW PERG.	NO. OF PERG. PERG.	NO. OF PERG. PERG.
	no.	gm	gm	ct	ct	ct			
PGA deficient rats									
13 days before breeding	15	218	— 12	13	100	0			
Day of breeding	13	229	+ 24	0	100	0			
	13	208	+ 20	0	100	0			
	11	225	+ 14	0	100	0			
	10	223	+ 23	0	100	0			
PGA-supplemented rats									
Day of breeding (13 days prior de- ficiency)	15	247	+ 96	0	7	93	104	1	74
Day of breeding	11	233	+ 119	0	0	100	115	5	107
	10	223	+ 114	0	0	100	111	2	104

<sup>1</sup> Data determined during the 20 days following mating.

TABLE 4  
Effect of food restriction and PGA supplementation on reproduction in the presence of the antagonist

EXPTL. GROUP	DATA BREED	BODY WT. AT MATING	WT. CHANGE DURING GESTATION <sup>1</sup>	AV. DAILY FOOD INTAKE <sup>2</sup>	FAILED IMPLAN- TATION %	RESORPTIONS OF IMPLAN- TATIONS	LITTERS OF IMPLAN- TATIONS	TOTAL YOUNG	YOUNG DEAD	AV. YOUNG PER LITTER	AV. WT. YOUNG
PGA- deficient	10	223	+ 23 ± 6.9 <sup>3</sup>	14.0 ± 0.5	0	100	0				
PGA- supplemented pair-fed controls	10	224	+ 79 ± 8.9	14.0 ± 0.5	0	0	100	105	1	9.0 ± 0.7	5.4
PGA- supplemented ad libitum controls	10	223	+ 114 ± 6.3	17.9 ± 0.6	0	0	100	111	2	10.4 ± 0.4	5.8

<sup>1</sup> Data determined during the 20 days following mating.

<sup>2</sup> Data determined during the 22 days following mating.

<sup>3</sup> Standard deviation of the mean.

*Effect of food restriction on reproduction in the presence of the antagonist*

To eliminate the factor of undernutrition as a cause of this marked reproductive failure in the presence of the antagonist, pair-fed controls were added in line with the above experimental procedure. The rats were carefully paired in regard to age and body weight on the day of breeding, as follows: PGA-deficient group, 101 days of age and 223 gm in body weight; pair-fed group, 106 days and 224 gm; ad libitum group, 105 days and 223 gm.

Table 4 presents the experimental data in detail. One hundred per cent of the PGA-deficient group resorbed, whereas the pair-fed group littered 100%. The restriction in food intake varied from 69 to 98% (average 79%) of the food eaten by rats given the PGA-supplemented diet ad libitum. The marked increase in efficiency of food utilization by the rats receiving the vitamin can be seen by comparing the weight changes during the 20-day gestation period, i.e., 23 gm for the deficient group and 79 gm for the pair-fed animals. Neither the deficiency nor the restriction in food intake during the gestation period interfered with implantation, as is shown by the normal number of implantation sites per rat for all groups. The food restriction did not affect the number of young per litter nor their average weight at birth. However, the group receiving the PGA-supplemented diet ad libitum gained much more during gestation than did the pair-fed group.

#### DISCUSSION

The data presented in this study demonstrate that pteroyl-glutamic acid is essential for reproduction in the rat when SST is incorporated in the diet. The deficiency produced in adult rats by the addition of SST to a purified diet lacking in the vitamin is only slight, as shown by the low incidence of resorptions (26% for 85 rats) and the length of deficiency prior to gestation required to produce the reproductive upsets.

The further addition of 0.5% of the chemical antagonist produces a very acute deficiency and reproductive failure occurs during the three-week gestation period. The normality of reproductive performance in PGA-supplemented controls restricted in food intake eliminates the factor of undernutrition and of other dietary deficiencies as a cause of these failures in reproduction. The lack of correlation between food intake and reproductive performance is further shown by the marked difference in reproductive success in the presence and absence of the antagonist with very little difference in food intake. In the presence of the antagonist 100% of the rats resorbed with an average 79% restriction, whereas in the absence of the antagonist 20% of the rats resorbed with an average 76% restriction in food intake. The limitation of food intake for either group was not as great as that previously reported for pantothenic acid deficiency during reproduction, i.e., 69% (Nelson and Evans, '46).

In regard to the specific function of pteroylglutamic acid in the reproductive mechanism of the rat, not much can be said at this time. Little interference with implantation was noted in the deficient groups except after prolonged periods of the deficiency prior to breeding. This is in marked contrast with the noticeable interference with implantation in the case of pantothenic acid deficiency (Nelson and Evans, '46) and that produced in pyridoxine deficiency with the aid of the potent vitamin antagonist, desoxypyridoxine (Nelson and Evans, '48). In pyridoxine deficiency, moreover, an early appearance of the erythrocyte sign was frequently observed. In this study the erythrocyte sign occurred normally on the 13th or 14th day of gestation, even for deficient animals receiving 0.5% of the antagonist. Furthermore, such animals gained weight up to the 13th day or even later and then lost slightly by the 20th day, so that a net gain in body weight resulted during the gestation period despite the simultaneous occurrence of resorption, a process practically completed by the 20th day as determined by examination of the uterus at autopsy. The findings of Hertz and Sebrell ('44) and

Hertz (45, 48a, 48b) that the characteristic tissue-growth responses to estrogen in the genital tract of both the female monkey and the chick require an adequate intake of pteroylglutamic acid are suggestive, as well as the recent report (Hertz, 48c) that the addition of the chemical antagonist (the same preparation used in this study) to a stock diet interferes with this response in the chick.

#### SUMMARY

Reproduction has been studied in adult rats placed on purified SST diets deficient in pteroylglutamic acid prior to the gestation period. In confirmation of previous studies, the impairment in reproductive performance was greatest after two months of deficiency prior to breeding; less marked effects were noted after three months of prior deficiency. There was little correlation between reproductive impairment and the age of the animals when the deficiency was started.

Pair-fed animals supplemented with the vitamin demonstrated that undernutrition was not the cause of resorptions under the prevailing experimental conditions. The level of synthetic pteroylglutamic acid given during gestation was not sufficient to counteract completely the effects of prior deficiency on the young, whereas young from mothers receiving the vitamin throughout the entire experimental period were in all respects normal.

The addition of 0.5% of a chemical antagonist markedly accentuated the vitamin deficiency and resulted in 100% resorptions for adult rats even without any prior deficiency period. The complete normality of pair-fed controls supplemented with high levels of the vitamin eliminated the factors of undernutrition and of other specific dietary deficiencies as causes for these reproductive failures.

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# THE MICROBIOLOGICAL ASSAY OF "FOLIC ACID" AS APPLIED TO MILK

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The folic acid content of cow's milk whey was reported by Wright, McMahan, Cheldelin, Taylor, Snell and Williams in 1941. Their data suggested that milk contained only a small amount of folic acid. Microbiological data published by Williams, Cheldelin and Mitchell ('42); by Wright, Skeggs, Welch, Sprague and Mattis ('45); and by Luekey, Briggs, Moore, Elvehjem and Hart ('45); and rat data obtained by Day, Wakin, Zimmerman and McClung ('46) support this suggestion. However, precise data for a number of milk samples have not been presented, the suggestion of Wright et al. ('45) that milk contains a large amount of "potential folic acid" has not been verified, and data concerning the lability of "folic acid" during the heat processing of milk have not been obtained. An investigation originally planned to clarify these three points developed into a critical study of the assay methods for "folic acid" as applied to milk. The results of that study are presented here.

## EXPERIMENTAL AND RESULTS

### *Methods and reagents*

Investigations in our laboratory support the theory that milk has a relatively low content of "folic acid" and does not contain any large amounts of "potential folic acid." The results of these investigations do not establish the exact

amount of "folic acid" in milk, although approximately 20,000 tubes have been filled, inoculated and titrated in microbiological assays which were designed to contribute either directly or indirectly to answering this question. We have not found the available methods sufficiently precise or specific to establish completely the average "folic acid" content of milk. In our laboratory when these methods were applied to the assay for "folic acid" in milk both *L. casei* and *Streptococcus faecalis* (American Type Culture Collection no. 8043) gave erratic results which were not at all comparable in precision to those obtained when these organisms were used for the assay of other vitamins or amino acids. When applied to milk the *L. casei* results were in general much higher than those secured by the use of *S. faecalis*, but were more variable, showed a greater tendency to drift, and were less reproducible than the *S. faecalis* results. The methods used were those suggested by Teply and Elvehjem ('45). We prefer the titrimetric methods since they permit the use of unclarified extracts for assay purposes. Synthetic pteroylglutamic acid was used as a standard in all our "folic acid" studies. Hog kidney conjugase was prepared according to the method of Bird, Robbins, Vandenbelt and Piffner ('46). The conjugase preparation was kept frozen until a few minutes before use.

A crude chicken pancreas conjugase preparation was made by grinding chicken pancreas with an equal weight of water in a Waring Blendor and filtering with the aid of celite through a no. 42 Whatman filter. A clear filtrate was obtained for the first portion of the extract filtered and this was frozen and stored for use. Later portions of the filtrate were cloudy and could not be clarified. The cloudy filtrate was less active and possessed a higher blank value. It was discarded.

### *Sample preparation*

In the preparation of liquid milk products for microbiological assay the simplest procedure is simply to dilute the sample so that the concentration of the unknown will fall in

the assay range. This is the procedure used for our first assays for "folic acid." In view of the well-known interference of fatty acids and other materials in unclarified extracts in the assay of riboflavin, pantothenic acid and biotin, we attempted to prepare clarified extracts of milk for "folic acid" assays. The casein in milk was precipitated by adjusting the pH to 4.5 with hydrochloric acid, the precipitated casein and occluded fat filtered off, an aliquot of the filtrate taken and brought

TABLE 1  
*Loss of "folic acid" during protein precipitation*

SAMPLE	TREATMENT	RECOVERY OF ADDED SYNTHETIC PTEROYLGLUTAMIC ACID	
		By <i>L. casei</i> method	By <i>S. faecalis</i> method
Fresh milk	1. Straight dilution	91	88
	2. Proteins precipitated at pH 4.5 and at pH 7.0	85	58
	3. Proteins precipitated with hydrochloric and metaphosphoric acids at pH 7.0	0	31
Evaporated milk	1. Straight dilution	102	101
	2. Proteins precipitated at pH 4.5 and at pH 7.0	62	31
	3. Proteins precipitated with hydrochloric and metaphosphoric acids at pH 7.0	0	35

to pH 7.0 with sodium hydroxide, and a second filtration made through a no. 42 Whatman filter paper. While this filtration gave reasonably clear filtrates in most cases, one could increase the filtration rate and insure an entirely clear final filtrate by making the first precipitation with a mixture of metaphosphoric and hydrochloric acids. These procedures proved to be unsatisfactory, however, because when pteroylglutamic acid was added to the milk before the protein precipitation, recovery experiments indicated that large losses occurred (table 1). The pteroylglutamic acid is probably

lost by adsorption on the precipitated protein, but this has not been proved.

Since the same discrepancy in results of the *L. casei* and *S. faecalis* assays occurred with dry skim milk powder as with fresh, evaporated, or dried whole milk, it did not appear that the discrepancy could be caused by the presence of fat in the milk extract. Nevertheless, in order to check this point further the trace of fat which occurs in dry skim milk was removed by repeated extraction with diethyl ether. The assay results were similar before and after extraction, and prove that the presence of fat in the extracts is not the cause of the great discrepancy between the *L. casei* and *S. faecalis* results.

### *Conjugase investigations*

A large number of assays were conducted after treatment of the milk samples with hog kidney conjugase. Preliminary treatments of milk samples with increasing amounts of hog kidney conjugase gave the same results for all increments and suggested that milk was free from inhibitors but that little or no "folic acid" conjugase was present in it. Later tests already reported (Hodson, '48) proved that hog kidney conjugase could be inhibited by protein and that for assay purposes the hog kidney conjugase treatment was of little value if carried out in the presence of relatively large amounts of protein.

Considerable trouble was experienced in arriving at optimum conditions for the hog kidney conjugase treatments. Since one of the problems encountered is common to other enzymatic treatments, it seems pertinent to discuss it briefly. This problem is that none of the commonly used preservatives prevented bacterial growth during the incubation period. The first treatment tried consisted of adjusting the pH of a diluted sample to 4.5, adding the desired quantity of the enzyme preparation, and incubating overnight at 45 or 37°C. The former temperature was used most often. After the overnight incubation the pH was adjusted to 6.8 and further di-

lution made if necessary. This procedure gave good recoveries and seemed satisfactory when applied to evaporated milk. With fresh or powdered milk samples it consistently gave erratic recoveries of added pteroylglutamic acid and results which were not reproducible. In an attempt to improve the enzyme treatment, fresh milk samples were incubated at pH 6.5-6.8 instead of 4.5 and at 37° C. Much higher values resulted from this change in pH. The samples were always curdled at the end of the overnight period but at first this was attributed to proteolytic enzymes in the hog kidney conjugase preparation.

This work was repeated and in the second trial some controls without enzyme but covered with toluene were incubated overnight at 37° C. and others were held overnight at 5° C. From the results of this trial, reported in table 2, it is apparent that covering the sample with toluene during the incubation did not prevent souring, and that bacterial synthesis of "folic acid" apparently takes place during the souring. It is possible that some bacterial growth and souring took place even in the samples held at 5° C. Bacterial growth and "folic acid" synthesis appear to have been limited at pH 4.5, but erratic recovery results in other experiments suggest this may not always be true when fresh milk is incubated with hog kidney conjugase for periods of several hours' duration. They suggest that the enzyme solution may introduce bacteria which multiply and increase the "folic acid" synthesis. Samples of high quality fresh milk (initial pH 6.6-6.8) have been treated with toluene, with benzene, with toluene and chloroform, and with benzene and chloroform and held overnight at 37° C. None of these preservatives prevented the souring of fresh milk. A preservative for this purpose should completely inhibit bacterial growth, should be easily inactivated or removed from the sample, should not react with "folic acid," should not react with or inactivate the conjugase, and should not react with other constituents of the sample or enzyme preparation to form products that will interfere in the enzymatic treatment or in the assay. A preservative

TABLE 2

*Error produced by bacterial growth during an overnight conjugase incubation period*

SAMPLE	TREATMENT	BACTERIAL GROWTH	"FOLIC ACID" BY S. FAECALIS ASSAY µg/l
A Fresh milk	1. Straight dilution held overnight at 5°C.	Suspected	5.5
	2. Incubated overnight at pH 4.5 without conjugase	Suspected	1.3
	3. Incubated overnight at pH 4.5 with conjugase	Suspected	1.0
	4. Incubated overnight at an initial pH of 6.6 without conjugase	Verified — milk soured and clabbered	21.7
	5. Incubated overnight at an initial pH of 6.6 with conjugase	Verified — milk soured and clabbered	more than 25.0
B Fresh milk	1. Straight dilution held overnight at 5°C.	Probably slight	1.0
	2. Incubated overnight at pH 4.5 without conjugase	Probably slight	0.5
	3. Incubated overnight at pH 4.5 with conjugase	Probably slight	0.6
	4. Incubated overnight at an initial pH of 6.6 without conjugase	Verified — milk soured and clabbered	8.6
	5. Incubated overnight at an initial pH of 6.6 with conjugase	Verified — milk soured and clabbered	17.6

meeting these requirements has not been found. The results reported in table 2 suggest the need for checking whether bacterial growth takes place during the incubation of other materials under toluene with conjugase, trypsin, and other enzymes.

Since a suitable preservative was not found, the procedure for enzymatic treatment was modified to minimize rather than eliminate bacterial growth. The length of the incubation period was reduced to one hour. A temperature of 45°C. was

TABLE 2

*Lack of isolatory effect of proteins on the activity of chicken pancreas conjugase*

PROTEINS TESTED	"FOLIC ACID" IN YEAST	
	By <i>L. casei</i> assay	By <i>S. faecalis</i> assay
	μg/gm	μg/gm
Yeast	0 <sup>1</sup>	0 <sup>1</sup>
Yeast plus chicken pancreas conjugase	14,270	13,167
Yeast plus casein (1:100)	0 <sup>1</sup>	0 <sup>1</sup>
Yeast plus casein (1:100) plus chicken pancreas conjugase	15,963	14,600
Yeast plus gelatin (1:100)	0 <sup>1</sup>	0 <sup>1</sup>
Yeast plus gelatin (1:100) plus chicken pancreas conjugase	14,132	14,519

<sup>1</sup> Some free "folic acid" is present in the yeast but this was not detected at the assay level used. The free "folic acid" is a small fraction of the total "folic acid" in this yeast sample.

used since this is optimum for the conjugases and above optimum for many bacteria found in commercial samples of fresh milk. Samples were diluted, prepared for assay and autoclaved as soon as possible after incubation with the enzyme. The hog kidney conjugase treatments were made at a pH of 4.5, which is below optimum for many bacteria. The chicken pancreas conjugase treatments were made at a pH of 7-8, since this enzyme is inactive at pH 4.5.

After it had been shown that the hog kidney conjugase was unsuitable for the determination of "folic acid" conjugates in milk products, extensive tests and assays were conducted



with the crude chicken pancreas conjugase preparation described above. Mims, Swendseid and Bird ('47) report that the chicken pancreas conjugase is not inhibited by nucleic acid as is the hog kidney conjugase. The data given in table 3 demonstrate that the chicken pancreas conjugase is not inhibited by protein. To test further for the presence of inhibitors in milk, mixtures of yeast and evaporated milk and pteroyl-triglutamic acid and evaporated milk were tested with *S. faecalis* before and after incubation with chicken pancreas conjugase. Before enzyme treatment the yeast and milk

TABLE 4

*Effect of chicken pancreas conjugase treatment on the "folic acid" content of evaporated milk*

SAMPLE NUMBER	"FOLIC ACID" IN RECONSTITUTED MILK			
	<i>L. casei</i> method		<i>S. faecalis</i> method	
	Without conjugase	With conjugase	Without conjugase	With conjugase
	$\mu\text{g/gm}$	$\mu\text{g/gm}$	$\mu\text{g/gm}$	$\mu\text{g/gm}$
1	16	23	2.1	2.7
2	9	15	0.8	1.2
3	4	4	0.7	0.9
4	16	16	1.3	2.0
5	18	18	1.1	1.2
6	10	14	1.4	1.8
Average	12	13	1.2	1.6

mixture give a value of 1.72  $\mu\text{g}$  per liter; after enzyme treatment the value increased to 7.75. Before enzyme treatment the pteroyl-triglutamic acid and milk mixture gave a value of 1.61; after conjugase treatment the value increased to 4.35  $\mu\text{g}$  per liter. These data clearly illustrate that the chicken pancreas conjugase is active in the presence of milk. In table 4 data on the "folic acid" content of evaporated milk before and after conjugase treatment are given. These suggest that a small amount of "folic acid" conjugate may be present in some samples of milk, but since the methods are not highly precise they can hardly be considered to prove this point com-

pletely. In any case it appears that no large amounts of the conjugated forms of pteroylglutamic acid, such as are found in yeast, are present in milk. The chicken pancreas conjugase has been used to treat a considerable number of milk samples since this treatment may help to give a slightly better understanding of the "folic acid" content of milk as determined microbiologically than methods measuring only "free folic acid." In table 5 data on the "folic acid" content of milk are given. A more extensive study was made of the "folic acid" content of evaporated milk, since samples from all parts of the United States were available for assay.

TABLE 5  
"Folic acid" content of milk  
(Assayed after treatment with chicken pancreas conjugase)

TYPE OF SAMPLE	NUMBER OF SAMPLES	"FOLIC ACID" IN FRESH OR RECONSTITUTED MILK			
		<i>L. casei</i> ASSAY		<i>S. faecalis</i> ASSAY	
		Average	Range	Average	Range
		$\mu\text{g/gm}$		$\mu\text{g/gm}$	
Fresh milk	12	24	11-74	1.6	0.9-2.4
Evaporated milk	52	13	2-29	1.6	0.8-3.3
Dry skim milk	11	18	13-26	1.4	0.7-2.2

### Sterilization losses

An investigation was made of whether or not there is a loss of "folic acid" activity during the sterilization of evaporated milk. Average results from 6 samples show no loss of "folic acid" activity as measured by the *S. faecalis* assay. Of the greater activity given by the *L. casei* assays, a loss of about 15% is shown. When 100  $\mu\text{g}$  of synthetic pteroylglutamic acid per a 14½ oz. can were added to evaporated milk, approximately 100% recovery after sterilization was obtained with either the *S. faecalis* or *L. casei* assays. The results showing the stability of "folic acid" during the sterilization of evaporated milk are in contrast to the large losses of up to 97% found by Cheldelin, Woods and Williams ('43) to

occur during the cooking of many foods and to the losses of 54 to 92% found by Schweigert, Pollard and Elvehjem ('46) to occur during the cooking of meats. Although because of the wide difference in actual values obtained by the *L. casei* and *S. faecalis* methods it is rather difficult to evaluate fully the results on the unfortified samples of milk, the results on the fortified samples show that pteroylglutamic acid is not destroyed by the sterilization treatment. These results are supported by the data of Daniel and Kline ('47), who found that a solution of crystalline folic acid could be autoclaved for 30 min. at 121°C. and at a pH of 6.8 without significant loss.

### *Chick assay*

Before the microbiological data compiled above were entirely collected it became apparent that it would not be easy to explain the discrepancy between the *L. casei* and *S. faecalis* results, and a small chick assay was planned to show whether or not any large amounts of "folic acid" might be present in conjugated or other forms available to the chick but not to bacteria. Shortly after the chick assay was completed, Lillie and Briggs ('47) published data obtained by a chick method showing that dry skim milk had a very low content of "folic acid," but their value is higher than is usually found with either microbiological method. When the writer recalculates their data to a reconstituted milk basis, a value of approximately 60 µg per liter is obtained. In our laboratory data in this range have, on a few occasions, been obtained with the *L. casei* method but never with the *S. faecalis* method.

The chick assay was performed by the method of Oleson, Hutchings and Sloane ('46). Ten chicks were fed the basal ration, and groups of 8 chicks each were fed each of the respective supplemented rations. The chicks were weighed weekly. At the end of the 4-week experiment hemoglobin determinations and feather and perosis scorings were made. The effect of pteroylglutamic acid on feathering has been shown by Oleson, Hutchings and Sloane ('46) and the effect

on perosis mentioned by Daniel, Farmer and Norris ('46). The anti-anemia effect was one of the first physiological properties of "folic acid" (vitamin Be) noted by Pfiffner, Binkley, Bloom, Brown, Bird, Emmett, Hogan and O'Dell ('43).

The sample of dry whole milk was especially prepared for the assay. It received no preliminary concentration or heat treatment except pasteurization before spray drying. Levels of 10 and 20% of dry whole milk supplements were used.

TABLE 6  
*Chick assay for "folic acid" in dry whole milk powder*

RATION	28-DAY DATA				
	Survival 4-28 days	Average weight	Average perosis score <sup>1</sup>	Average feathering score <sup>2</sup>	Average hemoglobin in blood
Basal	50	125 gm	2.0	16	5.6 gm/100 ml
Basal + 0.15 mg pteroylglutamic acid per kg	86	186	2.5	59	6.3
Basal + 0.5 mg pteroylglutamic acid per kg	100	273	0.3	83	8.6
Basal + 1.0 mg pteroylglutamic acid per kg	100	286	0.3	79	9.6
Basal + 10% dry whole milk	100	142	2.4	14	6.3
Basal + 20% dry whole milk	75	137	2.3	14	5.6

<sup>1</sup> Zero indicates no perosis, 4 indicates slipped tendon.

<sup>2</sup> Zero indicates no feathers, 100 indicates normal feathering.

Higher levels tend to unbalance the ration so that the physiological response may no longer be comparable to that shown on the standard rations. The use of a level as high as 20% may be questionable. This is especially true in the case of dry milk products, which have a flushing effect on chicks.

The data presented in table 6 cannot easily be used to assign a definite value for the "folic acid" content of the sample

under assay. Although the chick method is not sensitive enough to assay milk, it would appear to give results higher than those given by either the *L. casei* or *S. faecalis* methods but which approach more closely to those of the former.

#### DISCUSSION

In this report the term "folic acid" has been used very loosely to include any response of the test organism. Although this response was measured by comparison to a standard response produced by synthetic pteroylglutamic acid, it is apparent that in milk we may also be measuring other active compounds, and that the biological response may be altered by the presence of inhibitors and synergists. The fact that milk contains other active compounds, inhibitors or synergists may explain the lack of agreement in results secured by the various methods, since the response of *L. casei*, *S. faecalis* and the chick to these substances is known to vary.

There are many compounds that show stimulatory, substitute, or inhibitory effects for *L. casei* and *S. faecalis*. Some of these are not known to occur in nature, and the presence of any of them in significant quantities in milk has not been established. However, the presence of compounds of this type may well explain the discrepancy in results secured when the three methods under discussion are applied to milk. Stokes ('44) has discussed the folic acid activity of thymine and its nucleoside, thymidine. Krueger and Peterson ('45) have studied the response of *L. casei* and *S. faecalis* to thymine. Synthetic inhibitors have been discussed by Daniel, Norris, Scott and Heuser ('47); by Daniel and Norris ('47); by Franklin, Stokstad and Jukes ('47); by Hutchings, Mowat, Oleson, Stokstad, Boothe, Waller, Angier, Semb and Subbarow ('47); by Wooley and Pringle ('48); by Oleson, Hutchings and Subbarow ('48); by Hitchings, Elion, Vander Werff and Falco ('48), and by Strandkov and Wyss ('46). Colio and Babb ('48) reported on a stimulatory growth factor for *S. lactis R* (*S. faecalis*?). Daniel, Scott, Heuser and Norris ('48) have

reported a factor stimulating *L. casei*. Daniel, Scott, Norris and Heuser ('48) have provided information on the physiological activity of many synthetic pteridines and pyrimidines for chicks.

The reports of Shorb ('48), West ('48) and Riekes, Brink, Koniuszy, Wood and Folkers ('48) on vitamin B<sub>12</sub> may not be directly related to interference in "folic acid" assays, but they do point out the similar effect of vitamin B<sub>12</sub> and pteroylglutamic acid in promoting hemoglobin regeneration in human anemias. However, vitamin B<sub>12</sub> appears to be a more specific agent for the alleviation of Addisonian pernicious anemia. Ott, Riekes and Wood ('48) have reported that vitamin B<sub>12</sub> stimulates chick growth on a diet low in the animal protein factor.

It is thus obvious that there are a large number of compounds with physiological properties which overlap or counteract those of pteroylglutamic acid. Since the response to these compounds varies from organism to organism, it is obvious that it is difficult if not impossible to assess accurately the "folic acid" activity of all foods and feeds by comparison with a single standard such as pteroylglutamic acid. Some foods can be accurately assayed, others cannot. It is our experience that milk falls in the latter class.

#### SUMMARY

Data are presented which demonstrate that with the methods at present available it is difficult to arrive at an accurate estimate of the average "folic acid" content of milk.

Data secured by two microbiological methods and a chick technic are not in agreement, but those obtained by any of the methods support the conclusion reached by other investigators, that milk is a poor source of "folic acid."

#### ACKNOWLEDGMENTS

The author wishes to thank the officials of the Pet Milk Company for releasing this report for publication. He especially thanks Dr. E. A. Louder, Technical Director, for his part in this connection as well as his supervision of the research

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# THE EFFECTS OF THIOURACIL ON METABOLISM<sup>1</sup>

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Articles appearing recently in scientific journals indicate that under certain conditions of control, including the relative maturity of the experimental subject, there may occur definite increases in economy of body gain and improvement in carcass quality of swine and poultry when small amounts of thiouracil are included in the ration (Turner et al., '44; Muhrer and Hogan, '45; Kempster and Turner, '45; Andrews and Schnetzler, '46; Muhrer et al., '47; Van Der Noot et al., '47, '48). Andrews and associates ('47) found little or no effect on the growth of lambs but noted a trend toward a higher grade of carcass. A report by Beeson et al. ('47b) dealing with swine notes only loss of appetite and myxedema when the thionracil was added to the diet. Acevedo et al. ('48) report inferior gains resulting from the use of 0.25% thiouracil in the ration of growing swine fed ad libitum. Beeson and associates ('47a), working with steers, found that feed consumption was not affected and that there was a "tendency" for the thiouracil-fed animals to require less feed per pound of gain.

A reduction of 22% in CO<sub>2</sub> production in rats has been reported by Reineke and associates ('44), who supplied 0.1% thiouracil in drinking water. However, since their animals were fed ad libitum (personal communication), the CO<sub>2</sub> produced may have been a reflection of the amount of food consumed as well as of decreased metabolism caused by the drug.

<sup>1</sup> Authorized for publication on October 5, 1948 as paper no. 1477 in the Journal Series of the Pennsylvania Agricultural Experiment Station.



Even with equalized feed intake, greater storage of fat must be accompanied by a considerably higher respiratory quotient, making  $\text{CO}_2$  comparisons of little significance.

A study of these somewhat conflicting reports makes it appear that results are directly dependent on the level at which thiouracil is fed, the length of time during which it is given, and the age or stage of maturity of the experimental subject. It may be reasonably assumed from the evidence at hand that thiouracil should not be given to young rapidly growing animals, nor should it be fed for any extended period of time. It is of course known that the drug inhibits the function of the thyroid gland, and it is assumed that the efficiency of body gain results from a lowered basal metabolic rate and lesser activity. No reports have appeared, however, so far as the present authors are aware, in which actual measurements of body gains of fat, protein, and heat production have been made. The main objective of the work here reported was to establish the facts regarding the metabolic effects of thiouracil on the distribution of feed energy. A fundamental study of such a nature should be important as a background if thiouracil should come to have an important place in the economy of animal production.

#### EXPERIMENTAL

Three diets were prepared from a standard stock colony diet<sup>2</sup> in which the level of thiouracil<sup>3</sup> was the only variable. To the first, designated as the basal diet, none of the drug was added; to the second, 0.1% of thiouracil was added, and to the third, 0.2%. These diets were then compared in a 10-week growth, metabolism and body balance study. The experimental subjects were 24 male rats, selected as 8 sets of litter-mate triplicates. Each rat of a triplicate was assigned to one of the dietary groups, thus making three groups of 8 rats each. At the start of the experiment all rats were be-

<sup>2</sup> Rockland rat diet.

<sup>3</sup> The thiouracil (Deravet) was kindly furnished by the Lederle Laboratories, Pearl River, New York, through the courtesy of Dr. Mark Welsh.

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tracted with ether for a 48-hour period. The treatment of the ether extract and the body residue was as previously described. When the rats were killed at the end of the experiment, the thyroid glands were removed and weighed and the bodies were analyzed for moisture, ether extract, nitrogen, and energy. The total heat production for the 10-week period was computed by the body balance procedure; that is, as the difference between the gross energy of the food and the sum of the energy of body increase and of excreta.

During the course of the experiment, between the 4th and 6th weeks, measurements of the fasting heat production of all animals were made by the Haldane procedure. The animals were fasted for a 24-hour period immediately prior to the time when they were placed in the chambers where the respiration measurements were made during a period of at least 7 hours.

The energy content of the three diets was determined, by analysis, to be 4,037 cal. per gram, the nitrogen content 3.95%, and the moisture content, 8.39%.

## RESULTS

During the course of the experiment all of the refusals of feed were by the rats which received the thiouracil-supplemented diets. None of these refusals, however, occurred during the first 10 days of the experiment, thus making it apparent that the taste of thiouracil was not objectionable. Acevedo and associates ('48) recently reported a similar observation in working with swine. They noted that the thiouracil-treated group consumed feed in amounts similar to those of the control group during the first 10 days of the feeding trial and also that there was no apparent difference between the rates of gain of the two groups during the first 9 to 18 days.

While Andrews and associates ('47) have reported that swine, sheep and cattle apparently object to the taste of thiouracil, which results in decreased food intake, the authors of this paper believe that the taste of thiouracil is only of secondary importance when it is fed to rats. Of primary

importance is the fact that the feeding of thiouracil brings about a lowering of the animal's metabolism, and one of the first external manifestations of this condition is a depression of appetite.

In the experiment reported here the equalized food intake was sufficient to produce some growth and to permit valid comparisons of food utilization.

The average gains in body weight (table 1), excluding the contents of the alimentary tract, were 38.2 gm for the control rats, 57.0 gm for the rats which received 0.1% thiouracil, and 53.9 gm for the rats which received 0.2% thiouracil. Statistical treatment reveals that these differences are significant, with odds of 87 to 1 and 26 to 1, respectively.

TABLE 1

*Feed consumption, gain in body weight<sup>1</sup> of rats, and dry matter of body gain<sup>2</sup>*

RATION	FOOD EATEN (DRY MATTER)	GAIN IN BODY WEIGHT	DRY MATTER OF BODY GAIN
	gm	gm	gm
Basal	904.5	38.2	19.6
Basal + 0.1% thiouracil	904.5	57.0	28.6
Basal + 0.2% thiouracil	904.5	53.9	33.8

<sup>1</sup> Excluding contents of alimentary tract.

<sup>2</sup> Each datum represents the average of 8 rats during a period of 70 days.

The average values for dry matter of body gain were 19.6 gm for the control rats, 28.6 gm for the rats which received 0.1% thiouracil, and 33.8 gm for the rats which received 0.2% thiouracil. The odds that these differences are significant are 737 to 1 and 637 to 1, respectively.

The greater significance obtained by the statistical treatment of dry matter of the body gains as compared to a similar treatment of the gains in body weight can be attributed directly to differences in moisture content of the rat bodies. The average moisture content was 66.2% for the control group, 64.7% for the 0.1% thiouracil group, and 61.7% for the 0.2% thiouracil group. The significance of the difference between the first two groups is expressed by odds

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of 14 to 1, while the odds are 808 to 1 in the case of the first and third groups. The foregoing results demonstrate clearly that a consideration of gain in body weight without regard to possible variations in moisture content of the animal body may frequently lead to erroneous conclusions in the interpretation of the final results. In contrast to our results is the finding by Muhrer et al. ('47) that the carcasses of swine

TABLE 2  
*Distribution of feed nitrogen*<sup>1</sup>

RATION	FEED	FECES	DIGESTED	BODY GAIN
	gm	gm	%	gm
Basal	39.0	10.0	74.4	1.72
Basal + 0.1% thiouracil	39.0	9.5	75.8	1.84
Basal + 0.2% thiouracil	39.0	9.7	75.3	1.51

<sup>1</sup> Each datum represents the average of 8 rats during a period of 70 days.

TABLE 3  
*Distribution of feed energy*<sup>1</sup>

RATION	FEED ENERGY	FECES	DIGESTED	BODY GAIN			METABOLIZABLE	HEAT PRODUCTION
				Total	Protein	Fat		
	Cal.	Cal	%	Cal.	Cal.	Cal.	Cal.	Cal.
Basal	3896.0	1140.9	71.4	116.7	58.7	58.0	2654.0	2537.3
Basal + 0.1% thiouracil	3896.0	1119.5	71.9	196.2	62.9	133.3	2667.5	2471.4
Basal + 0.2% thiouracil	3896.0	1117.6	72.0	261.8	51.5	210.3	2667.2	2405.4

<sup>1</sup> Each datum represents the average of 8 rats during a period of 70 days.

which had received thiouracil contained 3% more water than did those of their controls.

The addition of thiouracil to the diet did not affect its metabolizable energy nor the digestibility of its nitrogen or the feed energy. The average digestibility of nitrogen (table 2) was 74.4%, 75.8% and 75.3%, and of the feed energy (table 3) 71.4%, 71.9% and 72.0% with the control, 0.1% thiouracil, and 0.2% thiouracil rats, respectively.

Concomitant with a decrease in heat production, the rats receiving thiouracil stored more energy than the control group. The average energy gains (table 3) were 116.7 Cal. for the controls, 196.2 Cal. for the rats which received 0.1% thiouracil, and 261.8 Cal. for the rats which received 0.2% thiouracil. The odds that the difference is significant between the controls and the rats on the lowest level of thiouracil are 575 to 1, and they are 2,499 to 1 that the difference is significant between the controls and the rats which received thiouracil at the 0.2% level.

The differences in energy gained as protein are not significant, while the differences in energy gained as fat are

TABLE 4

*Percentage distribution of feed energy and of metabolizable energy<sup>1</sup>*

RATION	FEED ENERGY		METABOLIZABLE ENERGY	
	Body gain	Heat production	Body gain	Heat production
	%	%	%	%
Basal	2.9	63.6	4.4	95.6
Basal + 0.1% thiouracil	4.9	62.0	7.4	92.6
Basal + 0.2% thiouracil	6.6	60.3	9.8	90.2

<sup>1</sup> Each datum represents the average of 8 rats during a period of 70 days.

highly significant, the odds being 141 to 1 that the 0.1% thiouracil group gained more energy as fat than the control group, and 3,665 to 1 that the 0.2% thiouracil group gained more energy as fat than the control group. Of the energy retained by the control group, 58.8 Cal. were gained as protein and 57.9 Cal. as fat; with the group which received 0.1% thiouracil, 62.9 Cal. were gained as protein and 133.3 Cal. as fat; and with the 0.2% thiouracil group, 51.5 Cal. were gained as protein and 210.3 Cal. as fat.

The percentages of metabolizable energy utilized for body gain (table 4) were 4.4% for the controls, 7.4% for the 0.1% thiouracil, and 9.8% for the 0.2% thiouracil groups.

The groups of rats which gained the most energy (the rats which received thiouracil) produced less heat than the con-



trol group, the three groups of animals metabolizing essentially the same quantities of food energy. The determined values for the average total heat production for the 10-week experimental period were: 2537.3 Cal. for the control group, 2471.4 Cal. for the 0.1% thiouracil group and 2405.4 Cal. for the 0.2% thiouracil group, or 65.1%, 63.4% and 61.7% of the food energy, respectively. The statistical significance of the difference between the first two groups is characterized by odds of 21 to 1, while odds of 999 to 1 indicate the significance of the difference between the control group and the group which received thiouracil at the 0.2% level.

Consideration of the relative magnitudes of the values shown in this table makes clear the fact that at any plane of nutrition the energy represented by heat production is always far greater than that represented by gain of body substance. Consequently, a given percentage reduction in heat production, if accompanied by a similar absolute gain in body tissue, signifies a much larger percentage increase in the latter.

In regard to the fasting heat production, calculated on an hourly basis and corrected to uniform live weight (200 gm), the differences between the control and thiouracil treated animals were even more significant than in the case of the total heat production for the entire 70-day feeding experiment. The average hourly fasting heat production for the control group was 758 Cal.; for the group which received 0.1% thiouracil, 673 Cal.; and for the group which received 0.2% thiouracil, 634 Cal. The odds are 737 to 1 that the control group had a higher fasting heat production than the group which received thiouracil at the 0.1% level, and 4,999 to 1 that the former group had a higher fasting heat production than their pair mates which received the diet containing 0.2% thiouracil. The control group produced 12.6% and 18.0% more heat than the 0.1% and 0.2% thiouracil groups, respectively, the corresponding values for the 10-week feeding experiment being 2.7% and 5.5%.

There were significant differences in the average weights of the thyroid glands at the conclusion of the experiment,

the average weight in milligrams for the controls, 0.1% thiouracil and 0.2% thiouracil animals being respectively as follows:  $10.4 \pm 1.00$ ,  $34.7 \pm 1.49$  and  $44.7 \pm 3.18$ . No histological examination was made of the glands, and the general appearance of the livers, lungs, hearts, and kidneys was normal.

The fact that the heat production of the fasting period was more significantly decreased by thiouracil than was the heat production of the feeding period suggests that this drug brings about increased economy of food utilization through a reduction in basal metabolism (and probably muscular activity) and that the true dynamic effect of the ration may be entirely unaffected.

#### SUMMARY

Three diets in which the level of thiouracil was the only variable were compared in a 10-week growth, metabolism, and body balance study using 24 male rats, selected as 8 sets of litter-mate triplicates, which were between 50 and 55 days of age when placed on experiment. Each rat of a given triplicate received identical quantities of food. The metabolizable energy of the ration was unaffected by thiouracil and no significant differences were found in the digestibility of protein or feed energy or in the body gains of protein. However, the rats receiving 0.1% and 0.2% thiouracil in their diets gained respectively 1.7 and 2.2 times as much energy as the rats on the same diet without thiouracil, these differences in body gain being mainly in the form of fat. Greater gain in body fat was accompanied by a corresponding decrease in heat production.

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# SIMULTANEOUS SURVEYS OF FOOD CONSUMPTION IN VARIOUS CAMPS OF THE UNITED STATES ARMY<sup>1</sup>

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FOUR FIGURES

(Received for publication February 7, 1949)

It is the purpose of this paper to record the results of simultaneous food consumption surveys conducted in 44 Army messes during the period 22 to 28 May 1945, inclusive. These surveys were carried out in compliance with an Army directive (ASF Circular No. 167, '45), for the purpose of reviewing the food requirements of troops. The study, together with other data, was used to form a sound basis for dietary recommendations in the Zone of Interior. The messes were chosen to be representative of the Army in the United States, and ranged from those serving soldiers performing sedentary duties to those serving soldiers undergoing rigorous training. This made possible procurement, under centralized control and standard methods, of Army food consumption data throughout the Zone of Interior, and distribution curves of these data are based on representative samples of food consumption at a given time. The information contained herein will add further to the studies made by Murlin and Hilde-

<sup>1</sup> The opinions expressed in this paper are those of the authors and do not necessarily represent the official views of any governmental agency.

brandt ('19), and Howe, Mason and Dinsmore ('19) in World War I, and by Howe and Berryman ('45) in World War II.

### METHODS

Each individual survey in the 44 messes was under the direct supervision of a Medical Department Nutrition Officer. These officers were scientific specialists originally commissioned directly from civilian life into the Army of the United

TABLE 1  
*Average quantities of food consumed in 44 Army messes during the period  
22 to 28 May 1945*

FOOD GROUPS	CONSUMED 1945	CONSUMED 1941-1943 <sup>1</sup>
	lb./man/day	lb./man/day
Meat, fish and poultry	0.834	0.909
Eggs	0.165	0.149
Milk and milk products (fluid milk equivalents)	1.260	1.016
Fats, butter and spreads	0.070	0.083
Fats, other	0.044	0.069
Sugar and syrups	0.280	0.262
Cereals and grain products	0.512	0.567
Beans, other legumes (dry), nuts (dry)	0.036	0.055
Vegetables, leafy green or yellow	0.302	0.359
Tomatoes	0.162	0.155
Citrus fruits	0.332	0.248
Potatoes	0.562	0.561
Vegetables, other than leafy green or yellow	0.248	0.252
Fruits, other than citrus	0.211	0.358
Fruits, dried	0.028	0.026

<sup>1</sup> Average food consumption in army messes as reported by Howe and Berryman ('45).

States, and had had considerable experience in conducting similar surveys prior to the beginning of this study. Since these data were obtained during the same 10-day period at all stations, and the Nutrition Officers conducting the surveys were operating in accordance with centralized instructions, it is felt that a high degree of comparability was obtained in the results.

Nutritional evaluation of the quantities of food consumed was carried out by the short method of evaluation of food groups described by Berryman and Chatfield ('43) and by Berryman and Howe ('44).

### RESULTS AND DISCUSSION

Table 1 shows the average quantities of the various food groups consumed in the mess per man per day during the

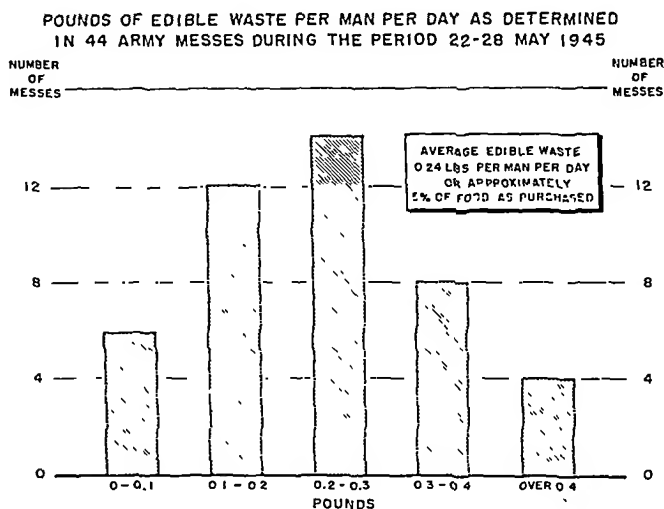


Fig. 1 Distribution curve, showing edible waste during simultaneous surveys of 44 Army messes. Ordinates represent number of messes with a particular value of food waste; abscissae represent food wastage in pounds per man per day.

period under survey; for comparative purposes, food consumption figures as reported by Howe and Berryman ('45) are shown in column 2 of this table. In order to determine the nutritional adequacy of the food consumed it was necessary to estimate not only the amounts of food prepared but also the amount of each food item wasted, and to subtract the nutrients contained therein from the nutrients in the food prepared. To obtain data on food wastage, all plate waste was segregated by food groups and weighed, and all kitchen

waste was also segregated and weighed. The range of food wastage in the different messes as shown by our data in figure 1 was from 0.08 lb. to 0.66 lb. per man per day, resulting in an over-all average of 0.24 lb. of edible food per man per day or approximately 5% of the food issued by the Quartermaster. This figure, as compared to an average edible food waste of 0.38 lb. per man per day reported in 1919, and of

TABLE 2

*Calculated nutritive value of average food consumed per man per day as determined in 44 Army messes during the period 22 to 28 May 1945*

NUTRITIVE FACTORS	CONSUMED	STANDARD DEVIATION	NRC <sup>1</sup>
Calories	3,744	299	3,000
Protein (gm)	127	11	70
Fat (gm)	181	28	
Carbohydrates (gm)	411	54	
Calcium (gm)	1.3	0.2	0.8
Phosphorus (gm)	2.2	0.2	
Iron (mg)	23.3	2.1	12.0
Vitamin A (I.U.)	10,042	1,960	5,000
Thiamine (mg)	1.6	0.2	1.5
Riboflavin (mg)	2.9	0.4	2.0
Niacin (mg)	23.0	2.4	15.0
Ascorbic acid (mg)	89.0	12.6	75

<sup>1</sup> 1945 National Research Council recommended daily allowance for a moderately active man.

0.39 lb. in 1941 and 0.32 lb. in 1943 reported by Howe and Berryman ('45), indicates the creditable strides made by the Army on food conservation.

During this study data were also obtained on messing practices, i.e., total elapsed preparation times. The methods of cooking and preparation of food were satisfactory in the majority of mess halls. In some instances, however, improper preparation and poor cooking practices were employed, resulting in lowered nutritive value of the food consumed. The

most common infraction of good cooking practices was poor timing of food preparation: meat was cooked and salads prepared too far in advance. Another factor, also having a bearing upon the amount of food left over, and wasted, was the overdrawing and underdrawing of rations. A comparison was made of rations drawn with the number of men actually fed, based on head counts made by each Nutrition Officer, and

TABLE 3

*Caloric value of food consumed by different types of organizations in 44 Army messes during the period 22 to 28 May 1945*

TYPE OF UNIT	DEGREE OF DUTY ACTIVITY	MESSES SURVEYED	AVERAGE VALUE OF FOOD CONSUMED
		no.	Cal./man/day
Armored	Training	3	3,868
Engineer	Training	3	3,726
Field artillery	Training	5	3,722
Infantry	Training	15	3,828
Military police	Duty	3	3,828
Quartermaster	Training	2	4,078
Signal	Training	1	3,507
Transportation	Training	1	3,676
Miscellaneous			
Headquarters detachment	General duty	6	3,499
Hospital patient mess	Hospital	3	3,729
Basic training	Training	2	3,471

a compilation of the rations drawn. Of the messes surveyed, 79% over-drew rations and 20% under-drew rations. Overdrawing rations to the extent of 5% was the average for the 44 messes surveyed.

The approximate nutritive value of the food consumed for the period is shown in table 2. It will be noted that the quantities of essential nutrients as calculated are liberal, and exceed, in every instance, the National Research Council's recommended dietary allowances for moderately active men.



The various organizations and units surveyed were of widely different composition and included a broad range of duties and varying levels of physical activity. Included were artillery, cavalry, engineer, infantry, medical, military police, quartermaster, signal, tank transportation, replacement center and miscellaneous troop units. The average caloric value of food consumed by the various types of units, as shown by our data in table 3, ranged from 3,471 Cal. for two basic training units, to 4,078 Cal. for two quartermaster units, with a total average of 3,744 Cal. per man per day. These figures indicate there was no significant variation of caloric intake with degree of activity of troops. Our estimated average caloric intake (3,744) agrees closely with the estimate reported by Howe and Berryman ('45) in World War II (3,694) and the findings of Murlin and Hildebrandt ('19) in World War I (3,633). The observation by Howe and Berryman that the calculated caloric value of food consumed in the mess rarely lies outside of the range of 3,000 to 4,000 Cal. is corroborated by our data presented in table 3.

Distribution curves showing the calculated nutritive values of the average amount of food consumed per man per day during the study are shown in figures 2 to 4 inclusive.

By actual count, the total number of men served in all the messes during the period of survey is as follows: breakfast 77,441; dinner 85,185; and supper 72,344. It is interesting to note the disparity between the number of men served breakfast, dinner, and supper. Undoubtedly the mess-hall diet was

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Fig. 2 Distribution curves, showing nutrients consumed during simultaneous survey of 44 Army messes. Upper left, *calories*: ordinates represent per cent of men with a particular value of caloric intake; abscissae represent calories consumed per man per day. Upper right, *proteins*: ordinates represent per cent of men with a particular value of protein intake; abscissae represent proteins in grams consumed per man per day. Lower left, *fat*: ordinates represent per cent of men with a particular value of fat intake; abscissae represent fat in grams consumed per man per day. Lower right, *carbohydrates*: ordinates represent per cent of men with a particular value of carbohydrate intake; abscissae represent carbohydrates in grams consumed per man per day.

NUTRIENTS CONSUMED PER MAN PER DAY AS DETERMINED  
IN 44 ARMY MESSES DURING THE PERIOD 22 -- 28 MAY 1945

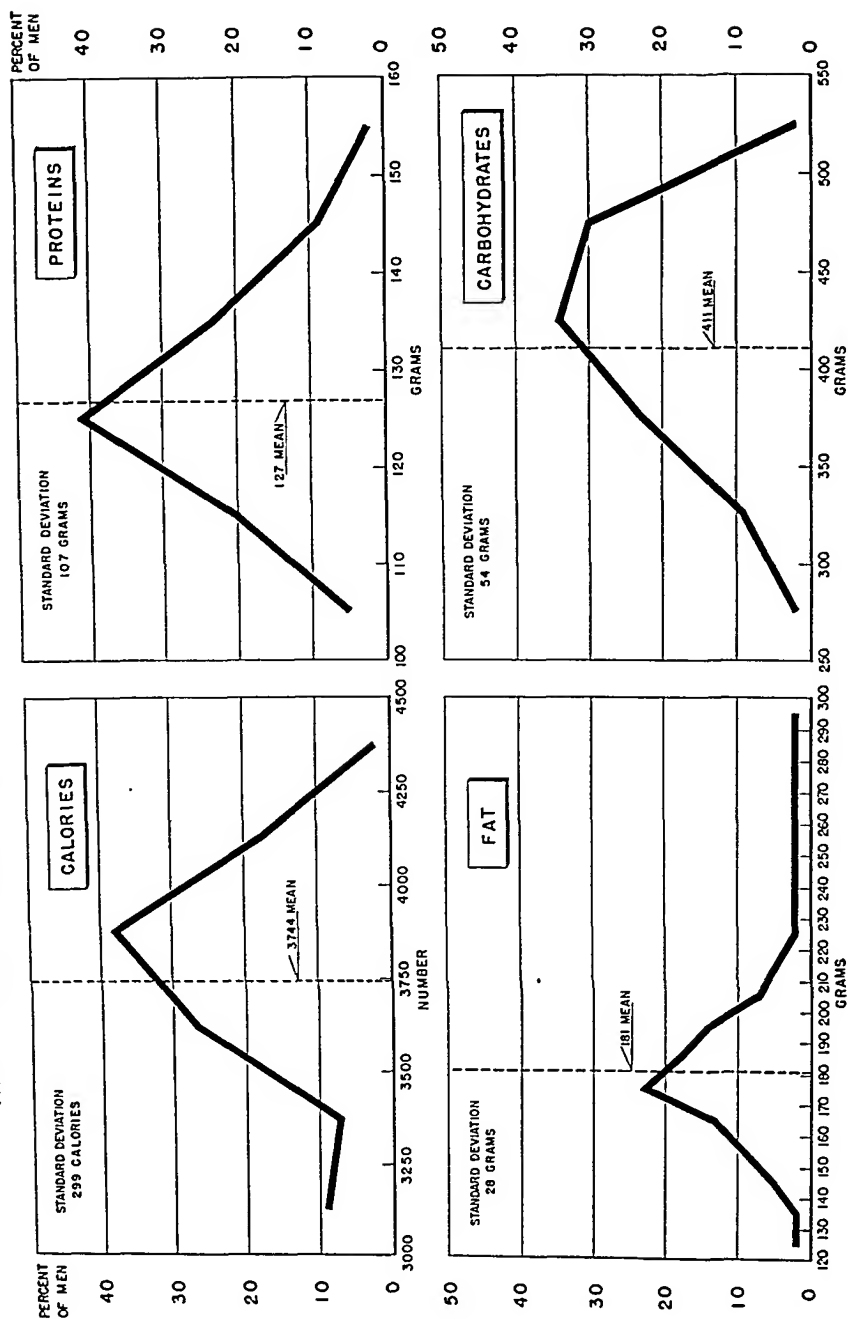


Figure 12

supplemented by food purchased at post exchanges, restaurants in nearby towns, and personal packages received by the individual soldier from relatives and friends. For reasons adequately listed by Murlin and Hildebrandt ('19), it is almost impossible to obtain an accurate record of the items consumed by the soldier outside the mess-hall. However, as the result of a nutritional survey of 261 canteens made in 1919, Murlin and Hildebrandt reported that the caloric value of food purchased at canteens averaged approximately 365 Cal. per man per day. McCay et al. ('44), reporting on sales by ship's canteens, estimate the average caloric value of food per man per day bought there to be approximately 400 Cal. On the basis of these findings it would appear justifiable to place the average soldier's daily caloric intake in the range of 4,000 to 4,100 Cal. i.e.,  $3,700 + 400$ . Cognizance should be taken, however, of the limitations applicable to this supposition. It is obvious that certain individuals may eat considerably more and others considerably less than the averages calculated above, both in the mess-hall and outside.

The minimum, maximum and mean temperatures prevailing at the various posts, camps and stations during the period of survey have been compiled for statistical treatment in order to determine their correlation with caloric intake. In different posts maximum temperatures of from  $60^{\circ}$  to  $96^{\circ}\text{F.}$ , minimum temperatures of from  $39^{\circ}$  to  $67^{\circ}\text{F.}$ , and mean temperatures of from  $54^{\circ}$  to  $79^{\circ}\text{F.}$  were recorded. Scatter diagrams of the various temperatures plotted against caloric intake showed no apparent correlation.

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Fig. 3 Distribution curves, showing nutrients consumed during simultaneous survey of 44 Army messes. Upper left, *calcium*: ordinates represent per cent of men with a particular value of calcium intake; abscissae represent calcium in grams consumed per man per day. Upper right, *phosphorus*: ordinates represent per cent of men with a particular value of phosphorus intake; abscissae represent phosphorus in grams consumed per man per day. Lower left, *iron*: ordinates represent per cent of men with a particular value of iron intake; abscissae represent iron in milligrams consumed per man per day. Lower right, *vitamin A*: ordinates represent per cent of men with a particular value of vitamin A intake; abscissae represent vitamin A in International Units consumed per man per day.

NUTRIENTS CONSUMED PER MAN PER DAY AS DETERMINED  
IN 44 ARMY MESSES DURING THE PERIOD 22-28 MAY 1945

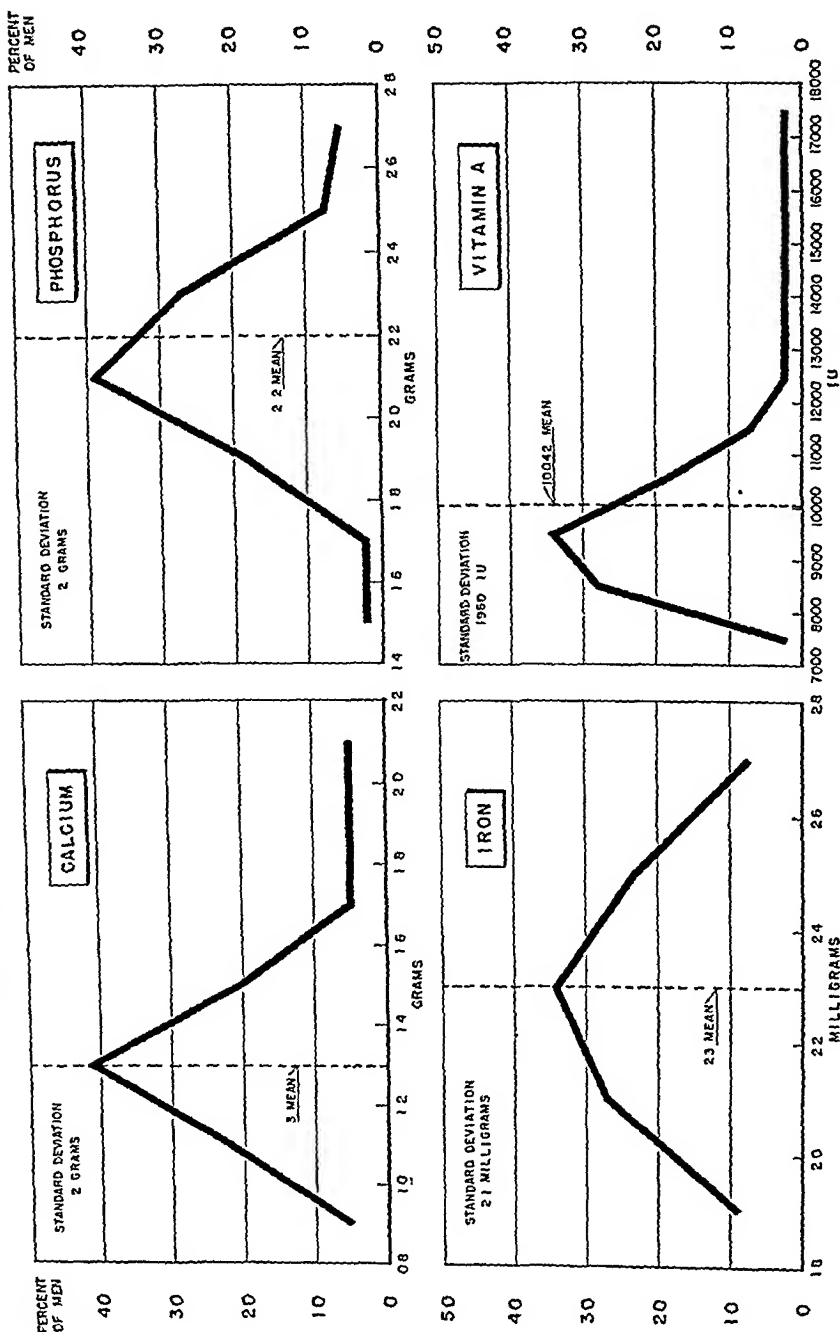


Figure 3

# NUTRIENTS CONSUMED PER MAN PER DAY AS DETERMINED IN 44 ARMY MESSES DURING THE PERIOD 22-28 MAY 1945

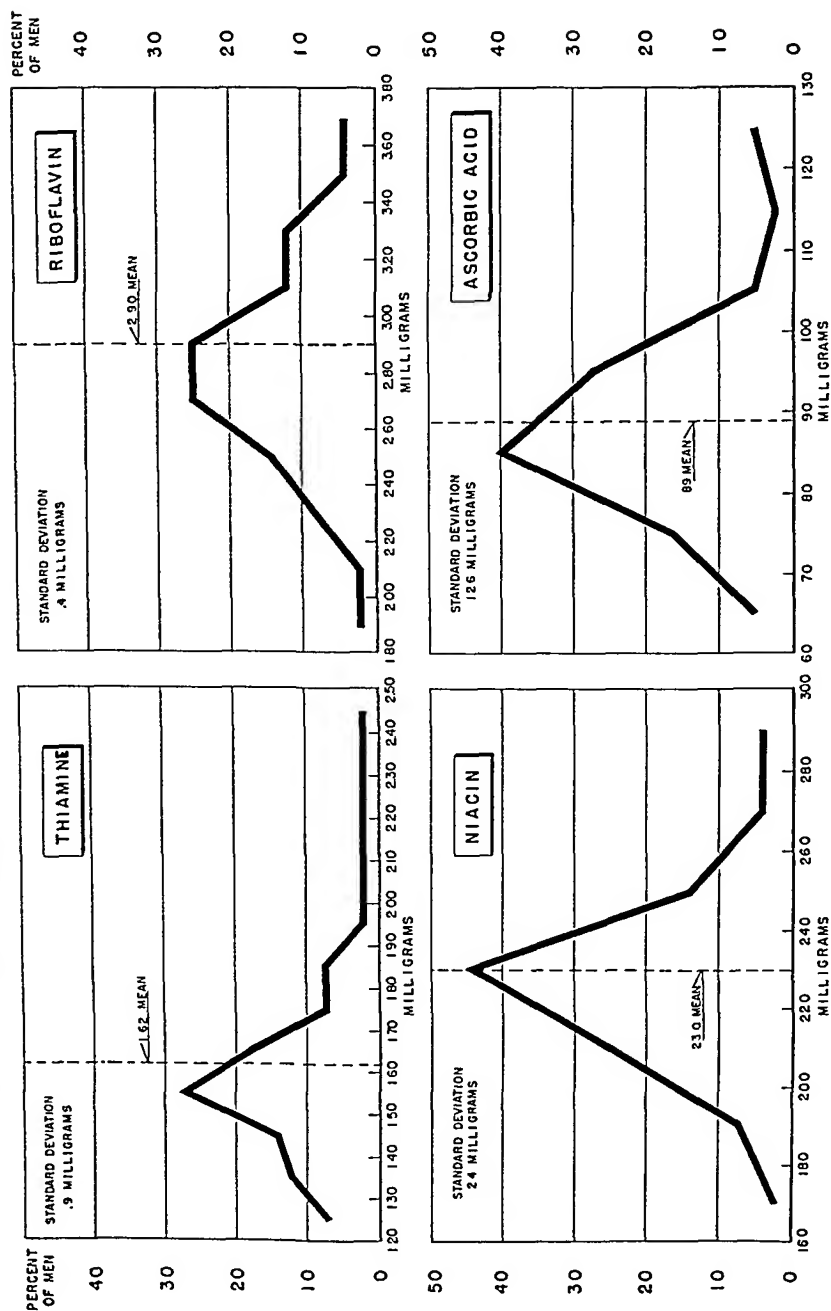


Figure 4

The 44 posts, camps and stations involved had a wide divergence of elevation, ranging from 10 ft. to 6,105 ft. above sea level. An attempt was made to determine a possible correlation between elevation and caloric intake. Elevations at the various posts were plotted against caloric intake at each post and analyzed statistically. In this particular instance no apparent correlation existed between these factors.

#### SUMMARY

Simultaneous nutrition surveys were carried out in May, 1945 at 44 military posts of the United States Army where Field Ration A, the rough equivalent of a liberal civilian diet in which there is a wide range of food, was provided. The food consumption levels reported indicated that, judged by National Research Council Standards, the American soldier in the Zone of Interior received a nutritionally adequate diet, although mess operations could have been improved through continued education and supervision of mess personnel. In the mess the average caloric intake of the average soldier per day, as determined by evaluation of food consumed, was approximately 3,700 Cal., fortified by an additional 300 to 400 Cal. consumed per day by virtue of food purchases at the post exchange. Food wastage comprised an average of 0.24 lb. of edible food per man per day. No significant correlations were found between the consumption of any individual nutrient and local mean temperature, altitude or the activity of the group.

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Fig. 4 Distribution curves, showing nutrients consumed during simultaneous survey of 44 Army messes. Upper left, *thiamine*: ordinates represent per cent of men with a particular value of thiamine intake; abscissae represent thiamine in milligrams consumed per man per day. Upper right, *riboflavin*: ordinates represent per cent of men with a particular value of riboflavin intake; abscissae represent riboflavin in milligrams consumed per man per day. Lower left, *niacin*: ordinates represent per cent of men with a particular value of niacin intake; abscissae represent niacin in milligrams consumed per man per day. Lower right, *ascorbic acid*: ordinates represent per cent of men with a particular value of ascorbic acid intake; abscissae represent ascorbic acid in milligrams consumed per man per day.

## ACKNOWLEDGMENTS

The food consumption values listed in this paper represent a summary of the data reported by and vouched for by Nutrition Officers carrying out nutritional surveys at various posts, camps and stations within this country. We wish to acknowledge the careful and diligent efforts of these officers which made this paper possible. Special acknowledgment is made to Captain Albert J. Dyer, under whose guidance the simultaneous surveys were so successfully completed.

Thanks are due to Dr. Robert E. Johnson, Director, Medical Nutrition Laboratory; Captain Denver I. Allen, Nutrition Branch, Surgeon General's Office; and Colonel Paul E. Howe, Nutrition Consultant, for their helpful suggestions and criticisms.

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# FOOD CONSUMPTION OF SOLDIERS IN A SUBARCTIC CLIMATE (FORT CHURCHILL, MANITOBA, CANADA, 1947-1948)<sup>1</sup>

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ONE FIGURE

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Little is known about the food consumption of garrison troops in arctic and subarctic climates. Most previous work has been done on troops in the field, such as in the Prince Albert Trials (Kark, Johnson and Lewis, '45) and in Exercise "Musk Ox" (Kark et al., '48). The present series of surveys was undertaken primarily to obtain information on the nutrient intake of garrison troops receiving an abundant ration in a subarctic climate. Secondary purposes were to obtain information on nutritional requirements as judged by medical criteria, and on possible peculiarities of food tastes in cold climates.

A favorable opportunity was offered at Fort Churchill, Canada, because the garrison troops in the winter of 1947-1948 were receiving an abundant and varied ration of fresh and frozen foods (Canadian Army Arctic Ration Scale 7, with an issue of 5,500 Cal. per man per day) and were under medical observation throughout the winter.

<sup>1</sup>The opinions expressed in this paper are those of the authors and do not necessarily represent the official views of any governmental agency.



Fort Churchill is situated on the west coast of Hudson Bay at the mouth of the Churchill River, about 500 miles south of the Arctic Circle. It was chosen for this survey because of the extreme "windchill" prevailing throughout the winter and early spring; in fact, it has one of the most consistently severe "windchills" of any inhabited area in the world and loss of body heat in exposed personnel is consequently very great. Although the temperature does not go as low as in

TABLE 1

*Average winter weather conditions at Fort Churchill, Manitoba, Canada  
(10-year period, 1936-1946)*

MEASUREMENT	MONTH				
	Nov.	Dec.	Jan.	Feb.	Mar.
Mean temperature (°F.)	+ 6	— 11	— 19	— 17	— 6
Mean wind velocity (m.p.h.)	17	15	15	15	14
Mean windchill <sup>1</sup> (kg cal./m <sup>2</sup> /hr.)	1,440	1,680	1,820	1,765	1,585
Total precipitation (inches, water equivalent)	1.03	0.66	0.48	0.61	0.87

<sup>1</sup> As defined by Siple ('45), "windchill," or dry shade atmospheric cooling, is a measure of the quantity of heat which the atmosphere is capable of absorbing from one square meter of exposed surface in one hour. It is expressed in kilogram calories per square meter per hour per degree Centigrade (0.369 B.T.U. per square foot per hour per degree Fahrenheit). The nomogram from which values are taken is based on the cooling rate upon a body at a neutral skin temperature of 33°C. (91.4°F.).

many places farther north, the average wind velocity is 15 to 20 miles per hour, and blizzards up to 50 miles per hour are quite common. Table 1 gives average winter conditions at Fort Churchill for the 10-year period from 1936 to 1946.

#### METHODS

In order to obtain data in early winter, mid-winter and late winter, surveys were conducted from the 9th to 18th of November, 1947; the 15th to 24th of February, 1948; and the 11th to 20th of April, 1948.

The surveys were conducted in a mess in which an average of 100 men ate all their meals. The military duties of these men included routine administration and general camp maintenance. Because of the great variety of duties of each man during the winter, it was impractical to secure a uniform record of activity. However, judging by interrogation and observation, the average man was moderately active and spent about three hours daily in the open.

In the conduct of the surveys, inventories were taken at the beginning and the end of each 10-day period, records were kept of food issued, plate waste and kitchen waste were weighed, and head counts were made at each meal. Computations were made by the short methods of Berryman and Chatfield ('43) and Berryman and Howe ('44), with the aid of the U. S. National Research Council's tables of food composition ('45).

At each meal a questionnaire listing all foods and beverages sold in the canteen (post exchange in U. S. Army terms) was given to 6 men at random; on this each man reported his consumption of these items for the previous day.

## RESULTS AND DISCUSSION

### *Nutrient intake*

The average intake of the various nutrients during the three 10-day surveys is shown in table 2. In every case the intake equals or exceeds the daily allowance recommended by the U. S. National Research Council for active men ('48). The reason for this excellent showing was because, on the average, the soldiers ate large amounts of a well-balanced ration. No specially supplemented foods were issued, and vitamin pills were not an item of issue.

This was an adequate diet from the medical viewpoint. Our staff at Fort Churchill kept continuous weight records of 16 men (average weight of 157 lb. in December, 1947) and recorded an average gain of 3.4 lb. per man from December 4, 1947 to April 22, 1948.

*Caloric intake in relation to weather*

A mass of observations during World War I and World War II have led to the generalization by Johnson and Kark ('47) that the colder the weather the more North American troops want to eat. The results of the present surveys are in agreement with this generalization.

During the three surveys temperatures ranged from  $+31^{\circ}$  F. to  $-37^{\circ}$  F., windchill from 400 to 2200, wind velocity from

TABLE 2

*Nutrients in food consumed by troops at Fort Churchill, winter 1947-1948*

NUTRIENT (Average per man per day)	PERIOD		
	9-18 Nov. 1947	15-24 Feb. 1948	11-20 Apr. 1948
Calories (cal.)	5,290	5,650	5,250
Protein (gm)	165	166	173
Fat (gm)	221	261	250
Carbohydrate (gm)	677	677	591
Calcium (mg)	1,455	1,636	1,159
Phosphorus (mg)	2,736	2,813	2,687
Iron (mg)	33	30	33
Vitamin A (I.U.)	20,278	10,543	13,010
Thiamine <sup>1</sup> (mg)	2.50	2.30	2.66
Riboflavin <sup>1</sup> (mg)	3.45	3.38	2.95
Niacin <sup>1</sup> (mg)	28.8	20.4	31.5
Ascorbic acid <sup>1</sup> (mg)	139	100	124
Average number of men fed daily	95	111	77

<sup>1</sup> Corrected for assumed cooking losses.

6 m.p.h. to 50 m.p.h., daily precipitation from 0.00 inches to 0.14 inches of snow (water equivalent), and relative humidity from 70% to 100%. Figure 1 shows the total caloric intake (mess intake plus canteen purchases) in relation to weather conditions prevailing at the time of the surveys. The caloric intake was inversely correlated with the mean environmental temperatures prevailing at the times of the three surveys, and was directly correlated with the mean windchill. In other

words, the troops voluntarily regulated their food intake in relation to the severity of the outdoor weather.

### *Pattern of food intake*

Another important generalization by Johnson and Kark ('47) which emerged from observations in World War I and

#### CALORIC CONSUMPTION, TEMPERATURE AND WINDCHILL FORT CHURCHILL, WINTER 1947-'48

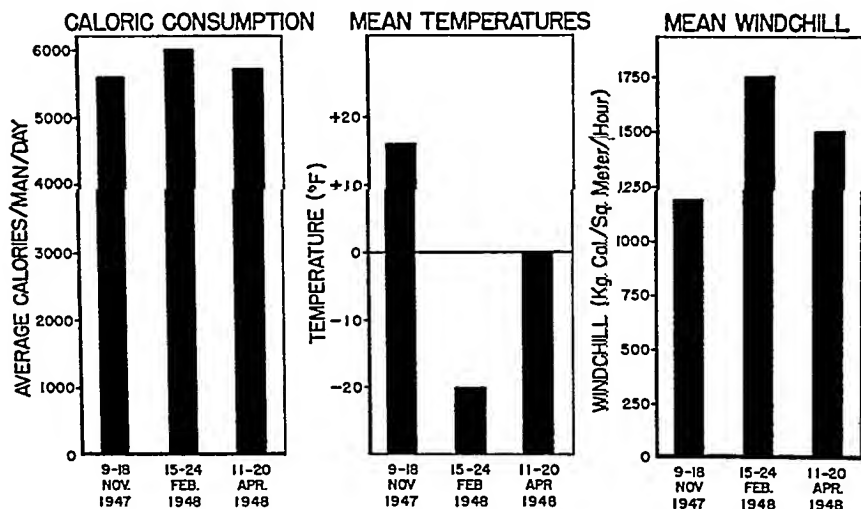


Fig. 1 Total caloric intake of troops at Fort Churchill in winter of 1947-1948 compared with mean windchill and mean temperatures prevailing at times of nutritional surveys. Ordinates, from left to right: Total average caloric intake daily per man. Mean temperature, expressed as degrees Fahrenheit. Windchill, expressed as kilogram calories per square meter per hour. Abscissae represent 10-day periods of each survey.

World War II was that in all climates the relative proportions of protein, fat, carbohydrate and vitamins consumed were surprisingly constant. The present surveys add further support to this generalization, particularly in relation to protein intake and actual consumption of food items.

Consumption of food items by groups is shown in table 3 for the present surveys and for United States soldiers in the

Zone of the Interior as reported by Howe and Berryman ('45) for 1943. There is only one group of foods in which the arctic ration was consistently lower than the United States garrison ration—milk and milk products. This situation was caused by a supply problem, the inability to secure fresh milk. Where the consumption of lard and other fats was low in the arctic

TABLE 3

*Average food consumption of soldiers at Fort Churchill, winter of 1947-1948, calculated by food groups*

FOOD GROUPS	FORT CHURCHILL			U. S. ARMY <sup>1</sup> CAMPS 1941-1943 (AVERAGE OF 455 SURVEYS)
	9-18 Nov. 1947	15-24 Feb. 1948	11-20 Apr. 1948	
	lb./man/day	lb./man/day	lb./man/day	lb./man/day
Meats, fish and poultry	1.023	0.980	1.272	0.909
Eggs, fresh	0.252	0.261	0.275	0.149
Milk and milk products	0.719	0.984	0.453	1.016
Butter	0.125	0.178	0.135	0.083
Fats, other	0.036	0.074	0.055	0.069
Sugars and carbohydrates	0.446	0.540	0.313	0.262
Cereals, grain products	0.966	0.922	0.911	0.567
Legumes	0.028	0.034	0.040	0.055
Vegetables, leafy green and yellow	0.846	0.248	0.430	0.359
Tomatoes	0.306	0.199	0.300	0.155
Fruits, citrus	0.317	0.179	0.222	0.248
Potatoes, white	0.990	0.948	1.197	0.561
Vegetables, other	0.511	0.465	0.508	0.252
Fruits, other	0.452	0.223	0.387	0.358
Cheese, Cheddar type	0.011	0.008	0.024	
Fruits, dried	0	0.027	0	0.026
Waste <sup>c</sup> (kitchen and plate)	0.75	0.51	0.38	0.32

<sup>1</sup> Data of Howe and Berryman ('45) for U. S. Army camps.

diet, it was offset by a higher intake of butter. Plate and kitchen waste were very high at first (0.75 lb./man/day) in comparison with U. S. Army camps listed in table 3 but after this situation was appreciated by the proper authorities steps were taken to reduce this figure, and by the end of the winter waste was down to a reasonable amount (0.38 lb./man/day).

Table 4 presents the average pattern of food consumption calculated by food groups and expressed as a percentage of the total food consumed. This type of calculation enables us to make a direct comparison between the patterns in U. S. Army training camps in temperate environments and those

TABLE 4

*Average patterns of food consumption of soldiers at Fort Churchill, winter of 1947-1948, calculated by food groups and expressed as percentage of total consumption<sup>1</sup>*

FOOD GROUPS	FORT CHURCHILL			U. S. ARMY CAMPS <sup>2</sup> 1941-1943 (AVERAGE OF 455 SURVEYS)
	9-18 Nov. 1948	15-24 Feb. 1948	11-20 Apr. 1948	
	%	%	%	%
Milk and milk products	10.1	15.7	7.0	20.0
Meats, fish and poultry	14.6	15.6	19.5	17.9
Cereals	13.7	14.7	14.0	11.2
Potatoes, white	14.1	15.1	18.3	11.1
Vegetables, leafy green and yellow	12.0	4.0	6.6	7.1
Fruits, other than citrus	6.4	3.6	5.9	7.1
Sugars and carbohydrates	6.3	8.6	4.8	5.2
Vegetables, other	7.3	7.4	7.8	5.0
Fruits, citrus	4.5	2.9	3.4	4.9
Tomatoes	4.4	3.2	4.6	3.1
Eggs, fresh	3.6	4.2	4.2	2.9
Butter	1.8	2.8	2.1	1.6
Fats, other	0.5	1.2	0.8	1.4
Legumes	0.5	0.5	0.6	1.0
Fruits, dried	0	0.4	0	0.5
Cheese, Cheddar type	0.2	0.1	0.4	0.0

<sup>1</sup> The data were analyzed statistically and showed no significant difference between the distributions.

<sup>2</sup> Data of Howe and Berryman ('45) for U. S. Army camps.

prevailing at Fort Churchill. Four points may be seen in the table. First, consumption of milk and milk products was low at Fort Churchill because of the supply problem mentioned above. Second, cereals and white potatoes comprised a higher percentage of the diet at Fort Churchill. Third, the sum of butter and other fats was not greatly different in the U. S.

army camps and at Fort Churchill. Fourth, there is no evidence of an increase in the appetite for any particular type of food during the winter. A statistical analysis of the data reveals no significant differences.

TABLE 5

*Percentage of caloric intake contributed by protein, fat, and carbohydrate in rations eaten by troops in various environments*

ENVIRONMENT AND PLACE	PROPORTION OF TOTAL CALORIES PROVIDED BY:		
	Protein	Fat	Carbo- hydrate
	%	%	%
<i>1. Arctic and subarctic areas</i>			
Fort Churchill, <sup>1</sup> 9-18 November, 1947	13	37	50
Fort Churchill, <sup>1</sup> 15-24 February, 1948	12	41	47
Fort Churchill, <sup>1</sup> 11-20 April, 1948	13	42	45
Exercise "Musk Ox," <sup>2</sup> February-May, 1946	13	42	45
<i>2. Temperate areas</i>			
U. S. Army Zone of Interior, <sup>3</sup> 1941-1943	13	43	44
U. S. Army Zone of Interior, <sup>4</sup> Mountain Troops, September, 1946	14	44	42
<i>3. Tropical areas</i>			
U. S. Army on Guadalcanal, <sup>5</sup> 1945	13	34	53
U. S. Army on Hawaii, <sup>5</sup> 1945	13	33	54
U. S. Army on Guam, <sup>5</sup> 1945	13	32	55
U. S. Army on Iwo Jima, <sup>5</sup> 1945	13	33	54
U. S. Army on Luzon, <sup>5</sup> 1945	12	34	54

<sup>1</sup> Data from present surveys.

<sup>2</sup> Data from Kark et al. ('48).

<sup>3</sup> Data from Howe and Berryman ('45).

<sup>4</sup> Data from Crowley, Johnson and Anderson ('47).

<sup>5</sup> Data from Kark et al. ('47).

Table 5 shows the per cent of Calories furnished by protein, fat, and carbohydrate in arctic, temperate and tropical diets. The close agreement between the different arctic and subarctic diets, and the close similarity among the arctic, subarctic and temperate diets, add further substantiation to the findings of Kark et al. ('48) from the Canadian Exercise "Musk Ox" that North American troops living in the winter in the arctic do not voluntarily consume a higher proportion of fat than

do troops in temperate climates. The small variation in the protein intake in the entire table is striking, and again corroborates a statement of Johnson and Kark ('47) that North American troops voluntarily consume about the same proportion of protein in the diet regardless of environment.

#### SUMMARY

A study was made of the voluntary food consumption of garrison troops at Fort Churchill, Manitoba, Canada, during 10-day periods in November, 1947, February, 1948, and April, 1948. Each of the three nutrition surveys was conducted on troops who ate together in the same mess and received an abundant ration of fresh and frozen foods, providing 5,500 Cal. per man per day (Canadian Army Arctic Ration Scale 7).

The average daily consumption per man, including canteen (post exchange) purchases, in the three surveys was 5,620, 5,590 and 5,690 Cal., respectively. The intake of all nutrients was well above the allowances for active men recommended by the U. S. National Research Council. Throughout the winter the troops were in good health and maintained their body weights.

The caloric intake in the three surveys was inversely correlated with the mean outdoor temperatures prevailing at the time of survey, and was directly correlated with the mean windchill.

In all three surveys the percentages of Calories furnished by protein, fat and carbohydrate remained almost constant, averaging 13, 41 and 46, respectively. These values are not significantly different from those reported many times for United States troops eating a garrison ration in temperate climates. As judged by the consumption of individual food items, the pattern of food habits was not different in the subarctic climate at Fort Churchill from that in U. S. Army training camps in temperate climates. In particular, there was no evidence of an increased appetite for fats in the subarctic winter.



## ACKNOWLEDGMENTS

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# THE PRODUCTION OF BIOTIN DEFICIENCY IN THE MOUSE<sup>1, 2</sup>

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TWO FIGURES

## INTRODUCTION

There are relatively few papers in the literature dealing with biotin deficiency in the mouse. In a recent review of the vitamin requirements of the mouse, Morris ('47) cited in this connection only the work of Nielsen and Black ('44), who found that better growth was obtained in mice on a synthetic diet when biotin was added than when it was omitted. Complete symptoms of the deficiency were obtained only when 0.6% sulfasuxidine was added to the diet. Morris considered these experiments to be not very clear-cut because of the poor growth response of the mice to the basal ration, since other investigators have obtained good growth in mice on similar basal rations without the addition of biotin and folic acid.

Lichstein, Waisman, McCall, Elvehjem and Clark ('45), in experiments to test the influence of biotin on the susceptibility of Swiss mice to experimental poliomyelitis, produced the

<sup>1</sup> Part of the material in this paper is from a thesis presented by Diane H. Winston in partial fulfillment of the requirements for the Sc.M. degree, Brown University, 1948.

<sup>2</sup> Supported in part by grants from the National Advisory Cancer Council and the Rhode Island Cancer Society.

<sup>3</sup> Horton-Hallowell Fellow of Wellesley College, 1947-48. National Cancer Institute Postdoctorate Research Fellow, 1948-49.

deficiency by including 20% powdered egg albumin in a synthetic diet. They report symptoms of deficiency like those that occur in the rat, appearing after 4 to 8 weeks on the diet. They had difficulty with diarrhea which they attributed to *Salmonella* infection, and suggested that mice deficient in biotin are more susceptible to naturally occurring infections than are animals maintained on a diet optimum in biotin. Since the diarrhea was fatal before the 12th day, it appears that it may have been due to an effect on the intestinal flora that precedes depletion of the biotin in mice. No effect of the deficiency on susceptibility to poliomyelitis was observed.

Kligler, Guggenheim and Herrnheiser ('46) in their study of biotin deficiency and resistance to infection used a diet containing egg white and found an increased susceptibility of mice to *Salmonella* infection. Since their experiments did not run over 24 days and they report none of the usual symptoms of the deficiency, it may be questioned whether the effect they obtained was due to the production of biotin deficiency or was merely due to an effect on the intestinal flora which may be directly influenced by a diet poor in biotin.

In our own experiments we have adapted for mice the diets used by various investigators for rats, and have consistently obtained the deficiency.

#### EXPERIMENTAL

The mice used were of a strain of albinos that is being developed in this laboratory. It is inbred and selected for vigor of growth and reproductive performance. The mouse room is kept at a constant temperature of from 75° to 78° F. The stock diet is a commercial chow<sup>4</sup> supplemented by rolled oats for nursing mothers and young up to 4 weeks of age. In the dietary experiments mice are kept in glass cages with floors of wire mesh raised from the bottom to minimize coprophagy.

For production of the deficiency several diets have been used. These are presented in summary in table 1. In all cases dried egg white has been included to insure the unavailability

<sup>4</sup> Purina Laboratory Chow.

of biotin, and yeast and cod liver oil have been used to supply the vitamins. We have used two types of egg white: a yellowish flake produced by drying fresh white of egg, and a powder produced by drying white of egg after fermentation to remove sugar. The latter corresponds to the imported "Chinese egg white" of earlier experimenters. It had been suggested to us that some of the avidin might be destroyed in the fermenting

TABLE 1  
*Composition of diets*<sup>1</sup>

INGREDIENT	DIET NO.					
	1D	1E	2A	5B	5C	9A
Vitamin-test casein (GBI <sup>2</sup> )	30	15	30	10	10	10
Egg white	..	15	..	20	20	20
Cornstarch (Argo)	..	..	29	29	14	28.5
Sucrose	47	47	..	..	15	..
Crisco	.	..	20	20	20	20
Corn oil (Mazola)	7	7	4	4	4	4
Cod liver oil (Squibb)	2	2	2	2	2	2
Powdered brewers' yeast (GBI)	8	8	8	8	8	8
Salt mixture USP XII, no. 2 (GBI)	4	4	7	7	7	7
Dried liver	2	2	..	..	..	..
Sulfaguanidine	..	..	..	..	..	0.5

<sup>1</sup> Several minor variants of these diets have been used in which Crisco replaced the corn oil, or sucrose all or a part of the cornstarch, or dried liver a part of the cornstarch, but no significant differences were observed.

We thank Henningsen Lamesa, Inc., for the egg white; Merck and Co., Inc., for the biotin; the Lederle Laboratory Div., American Cyanamid Co., for the sulfaguanidine; and Armour and Co. for the dried liver.

<sup>2</sup> General Biochemicals, Incorporated.

process, so that the use of the unfermented material might be desirable. We have made no quantitative studies but at the level we have been feeding egg white no significant difference between the two types is apparent. Inasmuch as the unfermented material must be refrigerated to avoid deterioration and must also be ground in a ball mill to assure its proper incorporation in the diet, we have found it more convenient to use the powdered fermented material.

In our first experiments a diet modified from that of Emerson and Keresztesy ('42) was employed. In all later experiments we have used a basal diet in general use in other investigations in this laboratory, differing primarily in that it contains a higher percentage of fat. This makes it easier to pack firmly in the food dishes and prevents to some extent the scattering of the food, although at best there is considerable wastage. It is also our feeling that the mice eat the diet with the higher fat content more eagerly and grow faster, which contributes to the early production of the symptoms. This is not in keeping with the observation of Nielsen and Elvehjem ('42) that fat seems to have a slight sparing action on biotin deficiency, particularly as far as the time of appearance of the paralysis is concerned. These workers fed to rats diets containing (a) no fat, (b) 5% corn oil, or (c) 41% lard with 10% egg white, and observed paralysis at 9, 9 to 12 and 12 to 14 weeks, respectively. Our rations contained 26% fat and 20% egg white, which may explain the difference in our experience.

We have used cornstarch rather than sugar as a carbohydrate in most experiments because there appeared to be no advantage in employing sugar in those experiments where it was used. Boas ('27) observed that potato starch gave some protection against egg white injury in rats, but that cornstarch had no such effect.

The dried liver was sometimes used during the first few days that the mice were on the diet to minimize the diarrhea which frequently occurs (Parsons, '31).

For cure of the deficiency symptoms we have injected biotin subcutaneously or pulped mouse liver intraperitoneally or have substituted the corresponding basal diet for the one containing egg white.

The biotin was given in a dose of either 2  $\mu$ g or 4  $\mu$ g a day. The liver was prepared by grinding the liver of one adult mouse in a glass tissue grinder with 2 ml of Ringer's fluid. A single injection of 0.5 ml of this mixture was the customary dose.

We have, in the last two years, conducted 20 different experiments using these various diets to produce biotin deficiency and have used over 150 mice. Since the purposes of the experiments have varied, and consequently the details, only two have been selected for detailed presentation.

*Experiment 167. Production of biotin deficiency on diet containing egg white*

- Nov. 7: Two litters of 8 mice each aged 16 days, weights 5.2 to 7.4 gm, placed on diet no. 5B with 2% dried liver.
- Nov. 9: Four developed diarrhea; three died and one recovered.
- Nov. 24: Changed to diet 5B without the dried liver.
- Nov. 29: (Twenty-two days on diet.) First symptoms in one animal, eyes swollen and much fur gone.
- Dec. 5: Practically no gain in weight since Nov. 29.
- Dec. 16: Symptoms generally developing.
- Jan. 9: Two animals had developed no symptoms and had continued to gain weight. All the rest had severe symptoms, including kargaroo posture in several. Seven mice used in another experiment. Four with mild symptoms retained.
- Jan. 22 to Feb. 19: The remaining animals, with severe symptoms, used in other experiments.

This experiment shows the variability in time of appearance and in severity of the symptoms, the early diarrhea, and the failure of two animals to develop any symptoms long after the rest had clearly done so. The average growth curve of these mice is shown in figure 1.

This figure shows the average growth curve of two mice on a control diet (2A) of the same composition except that the egg white was replaced by casein. Average growth curves of mice in two other experiments are also presented: Experiment 168 with 14 animals on the same diet used in experiment 167, and experiment 184 with mice on diet 5C, in which part of the cornstarch is replaced by sucrose.

*Experiment 285. Production of biotin deficiency in mice on a diet containing egg white and 0.5% sulfaguanidine*

- July 31: Four mice from one litter, aged 18 days, weights 9 to 10.7 gm, placed on diet no. 9A. No record of diarrhea.
- Aug. 19: Excellent gain in weight; weights now 21.7 to 26.6 gm.

- Sept. 2: Almost no additional gain in weight, animals all show loss of hair and swollen red skin around lower lip (33 days on diet).
- Sept. 20: Animals now losing weight; symptoms severe; 51 days on diet. Daily injections of  $2\mu\text{g}$  of biotin begun for three animals, the 4th being given none. All were retained on the diet.
- Sept. 27: Slight improvement—dose of biotin increased to  $4\mu\text{g}$ .
- Sept. 29: All treated animals gaining weight and eyes and skin improved.
- Oct. 1-4: Hair generally returning on all bare spots.
- Oct. 11: Untreated animal with extreme symptoms sacrificed for histological study.

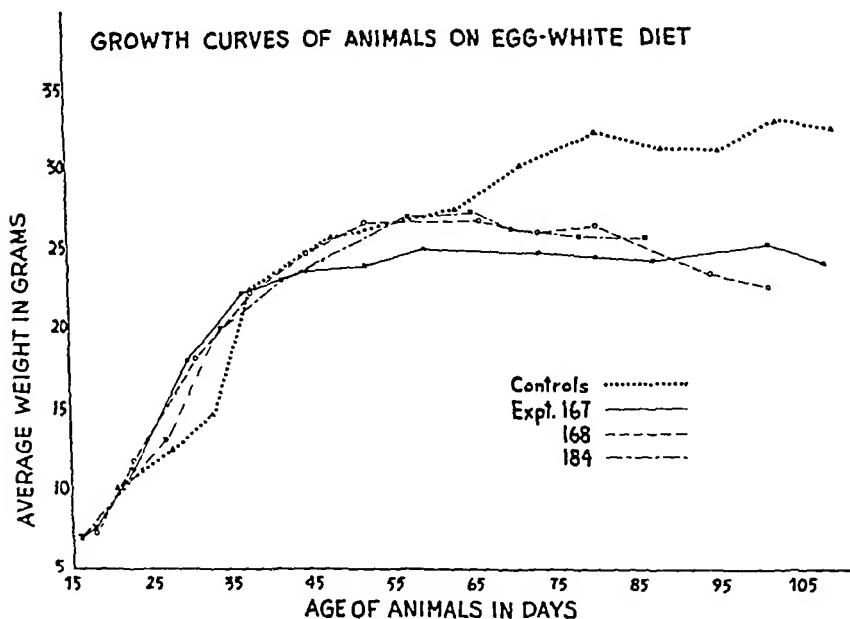


Figure 1

- Oct. 22: Treated mice look perfectly normal; female put with males.
- Oct. 24: Female has vaginal plug.
- Nov. 11: Female gave birth to 4 young which she failed to nurse.
- Nov. 13: One male sacrificed for histological study.
- Dec. 3: Female and male still thriving; sacrificed for study of intestinal flora and histology.

The individual growth curves of these mice are shown in figure 2. The occurrence of the symptoms was strikingly uniform. Histological study showed that there was no goitrogenic effect of the sulfaguanidine. Complete recovery result-

ing from injection of biotin while still on the diet indicates that there is no toxic effect of the drug and that the deficiency is a simple one.

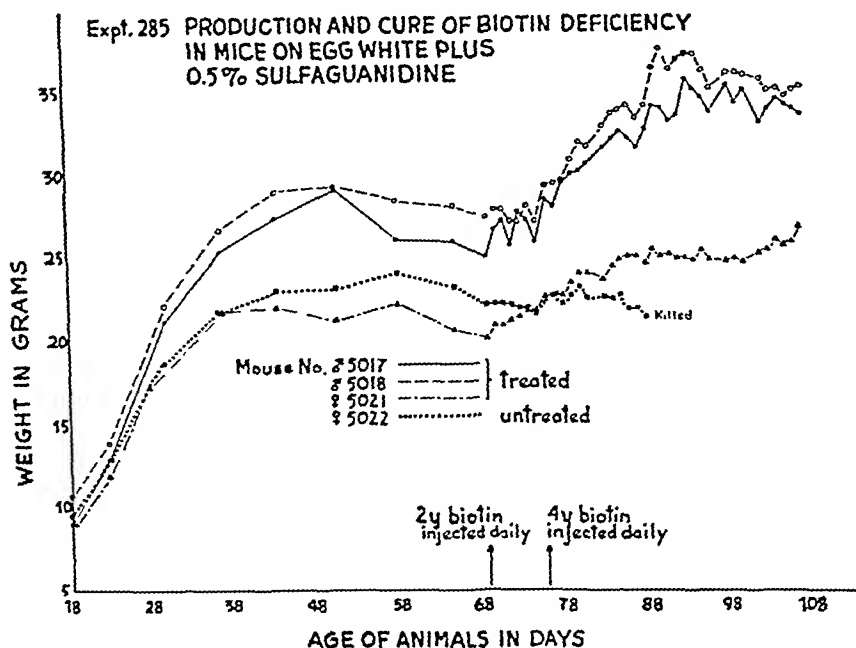


Figure 2

## DISCUSSION

We have obtained biotin deficient mice by feeding all of the diets in table 1 that contain egg white. At one time or another we have seen in the mouse most of the symptoms and phenomena described by others as occurring in the rat, and in all cases where a cure has been attempted it has been effected. In our more recent experiments with diet 5B using 16-day-old mice, recognizable symptoms begin to appear in three weeks and by 4 or 5 weeks the symptoms are extreme. The animals seem to grow well until about the time the symptoms begin to appear, when the growth curve falls off to a plateau (fig. 1). The excellent description by Sullivan and Nicholls ('42) and Sullivan, Kolb and Nicholls ('42) of the appearance



of the symptoms in the rat and their disappearance as curative treatment becomes effective is generally applicable to the mouse, with the single exception that the considerable accumulation of brownish seborrheal material on the skin which occurs in the rat seems to be absent in the mouse.

In a few animals the symptoms fail to appear. An animal without symptoms may be in a cage where the rest have severe symptoms. This seems in many cases to be due to coprophagy in spite of the raised wire mesh bottoms of the cages. The fact that the intestinal bacteria may synthesize large amounts of biotin has been suggested or shown by several workers (e.g., McGregor, Parsons and Peterson, '47).

In a few experiments we have used C<sub>57</sub> black mice. Several investigators have reported a graying of black hairs in rats on a diet containing egg white, but Kennedy and Palmer ('45) failed to obtain it in their experiments. In the C<sub>57</sub> black mice we observed that the hair becomes rusty and in some cases gray. As in the white mice, some areas of the skin become completely denuded. After the injection of 2  $\mu$ g of biotin daily for a few days, with the animals still on an egg white diet, new hair begins to grow out on the bare areas. While it may be gray at first, it ultimately becomes black and shining. Emerson and Keresztesy ('42), on the other hand, reported that rats rendered biotin deficient on a diet containing egg white showed a symmetrical graying of the ingrowing fur after treatment with biotin for 12 days and that, furthermore, the gray might persist through 5 weeks of treatment. They themselves point out, however, that the persistence of the "spectacle eye" condition may indicate that their dose was suboptimum. We would tentatively conclude, therefore, that biotin is directly or indirectly a necessary factor in the development of black hair color.

From reports in the literature it would appear that when the rat is used in the study of biotin deficiency, the production of the deficiency is more consistent than we have found it in the mouse. One source of variation in results may be the initial state of the experimental animals. Sullivan and Nicholls

('42) have summarized the evidence in the literature in favor of using very young animals, preferably from mothers on diets poor in biotin. Our best results have been obtained with young weaned as early as possible on the egg white diet, preferably on the 16th to the 18th day of age. The nature of the intestinal flora must also be a cause of variation, since it is normally a source of considerable biotin. Gall, Fenton and Cowgill ('47) have shown that the cecal flora of different strains of mice may be different. This may be the explanation of the unexpected behavior of the mice in the experiments of Nielsen and Black ('44), which on a biotin low basal diet that would ordinarily produce no deficiency showed marked increase in growth when biotin was added. They concluded that the mouse needs a dietary source of biotin. Fenton, Cowgill and Stone ('48), however, report that with their purified diets and under the conditions of their experiments they have not found that the mouse requires a dietary source of biotin or folic acid. To explain the discrepancy they suggest the possibility that the requirement for biotin and folic acid may be different in different strains. That the difference in requirement may be due to differences in cecal flora is an obvious possibility. The constancy of our results when the change in intestinal flora was controlled by the use of sulfa drugs, which is discussed below, is evidence of the importance of the intestinal flora as a source of variation.

A disturbing effect sometimes produced when the young mice are first placed on the egg white diet is a diarrhea which in a few cases may be fatal. This has been observed in rats by many workers (Parsons, '31; Parsons and Kelly, '33; and Salmon and Goodman, '34). In agreement with Bateman ('16), we have found no significant intestinal inflammation accompanying the diarrhea. On the other hand, Lichstein, Waisman, McCall, Elvehjem and Clark ('45) report an acute inflammation of the intestinal tract and enlargement of the spleen. It is generally agreed that the diarrhea is not due to biotin deficiency but to some other action of the egg white diet. It seems possible that it may be due to a change in the

intestinal flora as a result of the lack of available biotin in the food. Lichstein and his co-workers found large numbers of organisms in the feces which they identified as probably *Salmonella typhimurium*, which may account for the difference in our findings; in our animals the infection may have been a less virulent one. This would also account for the fact that our animals frequently recover and thrive for some time. The diarrhea has not occurred when sulfaguanidine has been used with the egg white in the diet. This would suggest that the coliform organisms are responsible for the diarrhea when it does occur, for it is the coliform population in particular that is reduced by the sulfa drugs (Evenson, McCoy, Geyer and Elvehjem, '46).

Several investigators have shown that the inclusion of the less readily absorbed sulfa drugs, sulfaguanidine and sulfasuxidine, in a semisynthetic diet produces a complicated deficiency in rats that can be prevented or cured by combined treatment with folic acid and biotin. The consensus is that this is due to a change in the intestinal flora. Evenson et al. ('46) have shown that the coliform organisms are almost eliminated after 5 days on a diet containing 0.5% sulfasuxidine. Gant, Ransone, McCoy and Elvehjem ('43) conclude that it is the coliform organisms that produce the biotin and folic acid in the intestine, and that this source makes the production of the deficiency irregular with egg white and prevents it on a diet low in biotin. Nielsen, Shull and Peterson ('42) have shown that synthesis of biotin is almost arrested in the rat on a diet low in biotin with 0.5% sulfaguanidine. In our experiments the sulfaguanidine has been added to a diet containing egg white which also contains yeast. This diet has been the most successful in the production of the symptoms in the mouse. After symptoms were completely developed, they were rapidly cured by injection of two to 4  $\mu$ g of biotin subcutaneously daily while the mice were still on the diet containing sulfaguanidine and egg white. One female mouse gave birth to a litter on this regimen. She had been on the diet

since the age of 18 days and had developed and recovered from a severe biotin deficiency.

The deficiency obtained with sulfa drugs when pure vitamin supplements without folic acid and biotin are used apparently involves deficiency not only in these two but also in pantothenic acid (Black, Overman, Elvehjem and Link, '42) and possibly vitamin K (Wright and Welch, '44). In our experiments, however, the deficiency is produced by supplying avidin in egg white which combines with the available biotin of the diet, and at the same time supplying sulfaguanidine which eliminates a large part of the biotin synthesis in the intestine. Since the symptoms are cured by the subcutaneous injection of biotin, the deficiency must be a simple one involving biotin alone. Emerson and Wurtz ('45) have used sulfasuxidine in conjunction with egg white to produce biotin deficiency in the rat. They also considered that a simple biotin deficiency was produced because the symptoms were prevented or overcome by the oral administration of 5  $\mu$ g of biotin daily.

The possibility of toxicity of the sulfaguanidine must be considered. It has been shown to be goitrogenic in rats but at a higher level than we have fed it (Mackenzie, Mackenzie and McCollum, '41). Black, McKibbin and Elvehjem ('41), however, found no harmful effects in rats receiving 0.5% in the diet. We have found none in mice after many weeks with 0.5% in the diet. It would be surprising if any goitrogenic effect appeared at this level in the mouse, since even such a vigorous goitrogenic agent as thiouracil must be used in the mouse at a level much higher than in the rat to produce the effect. Other pathological effects following the administration of sulfaguanidine have been described by Daft, Ashburn, Spicer and Sebrell ('42), Daft, Ashburn and Sebrell ('42), and Spicer, Daft, Sebrell and Ashburn ('42). These involve changes in arterial walls, muscles, and liver. Welch and Wright ('43), however, found no such effects and were unable to explain the discrepancy. Although we have made no systematic study, we have seen no evidence of such changes.

The fact that the animals recover from severe symptoms while still on the diet containing egg white and sulfaguanidine when biotin is injected daily, and that they thrive and grow and are able to reproduce, would seem to indicate not only that the deficiency is a simple one, but that the sulfaguanidine is not toxic when fed to mice at the level used. This would, then, seem to be the best method of producing biotin deficiency in the mouse quickly and certainly.

#### SUMMARY AND CONCLUSIONS

1. Biotin deficiency has been produced in mice by diets containing dried egg white with and without the addition of sulfaguanidine.
2. All of the symptoms described in the rat have been observed in the mouse except the accumulation of brownish seborrheal material on the skin.
3. The appearance of the symptoms of the deficiency have been variable on the egg white diet without sulfaguanidine. The sources of this variability are discussed. With sulfaguanidine the results have been consistent.
4. The fur of C<sub>57</sub> black mice becomes rusty or gray on the deficiency-producing diet. It becomes black when biotin is injected while the animals are still on the same diet. It is concluded that biotin is directly or indirectly a necessary factor in the development of black hair color.
5. The diarrhea which may occur soon after mice are placed on the egg white diet is attributed to a change in the intestinal flora.
6. Sulfaguanidine at the level used, 0.5% in the diet, is not toxic.
7. The deficiency produced by the combination of egg white and sulfaguanidine is a simple one since it can be cured by the injection of biotin while the animals are still on the diet.

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# THE WRIST STIFFNESS SYNDROME IN GUINEA PIGS

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In 1936 Bahrs and Wulzen reported a wrist stiffness in guinea pigs which they considered to result from the lack of an antistiffness factor in the diet. Since then this deficiency syndrome has been studied in detail by van Wagtendonk and Wulzen and co-workers ('43-'47) at Oregon State College and, more recently, by Oleson et al. ('47) and Petering et al. ('48).

The Oregon group has described an impressive list of symptoms which characterize this deficiency disease, among which are a stiffness of the wrists and elbow joints, extensive calcification of the soft tissues, alterations of the concentrations of various phosphorus and calcium compounds in tissues, a decrease in the serum alkaline phosphatase activity, and others.

Among the crude dietary supplements studied, raw sugar cane juice has been reported to be the richest source of a factor which prevents or cures this joint stiffness. A white crystalline compound was isolated from cane juice by van Wagtendonk and Wulzen ('46) which reportedly cured wrist stiffness when fed in 5 daily doses of 0.002  $\mu$ g each. In a more recent publication, Simonsen and van Wagtendonk ('47) reported that the activity of this crystalline material is actually due to traces of an impurity which, as pointed out by an anonymous writer in *Nutrition Reviews* ('48), means a factor active at



a level of the "order of 0.00002 microgram — a vanishingly small figure." Oleson et al. ('47), after finding that dried penicillin mold pad was curative and that this activity was found in the sterol fraction, tested 59 pure steroids and found that over 24 possessed some antistiffness activity. Of these compounds the esters of ergostanol were the most active. Petering et al. ('48) have reported confirmation of the Oregon work in part, i.e., the wrist stiffness. Ergostanyl acetate was found by these workers not to be as active as Oleson et al. had reported.

Two laboratories have published results which fail to confirm the Oregon work. Kon et al. ('46) and Homberger and Reed ('45) failed to demonstrate wrist stiffness in guinea pigs fed a skim milk diet (Wulzen and Bahrs, '41). Furthermore, these workers reported poor conditions of their animals and a high mortality, observations confirmed by Petering and colleagues ('48), Oleson and associates ('47), and Brown ('47). This high mortality on a skim milk diet led Petering and Oleson and their co-workers to use a different basal diet.

The present paper reports the results of our studies of this interesting but confusing syndrome.

#### MATERIALS AND METHODS

Two experiments are here reported involving over 200 guinea pigs. These guinea pigs, of mixed sexes and weighing 275–550 gm each, were obtained from a large local colony. They were divided at random into treatment groups and fed thereafter in individual wire screened cages. Five different diets were fed. Three of these diets were the same, insofar as was possible, as those described by the Oregon workers. Of these three, one was a *skim milk* diet (Wulzen and Bahrs, '41) supplemented with iron, copper, unsweetened orange juice, carotene, autoclaved oat straw and salt. Another was a *dry skim milk* diet supplemented with iron, copper, 14 vitamins and autoclaved straw. The *purified* diet (van Wagten-donk, Schocken and Wulzen, '44) included "vitamin-free" casein, cornstarch, irradiated yeast, salt mixture, orange juice,

mixed tocopherols and autoclaved straw. The irradiated yeast contained 9000 I.U. of vitamin D per gram and at the level fed (10%) furnished a large excess of this vitamin. The 4th diet, designated as the *Lederle diet*, consisted of natural feed-stuffs and was reported by Oleson et al. ('47) to produce a stiffness of the wrists. The 5th diet was a commercial *calf meal* which is widely used as a rat stock diet (Maynard, '30), supplemented with salt, autoclaved straw and ascorbic acid (7 mg per day). The phosphorus content of the calf meal was increased by adding 2% of  $\text{NaH}_2\text{PO}_4$ . The calcium and phosphorous contents of the purified, Lederle and calf meal diets were, respectively, 0.61% and 0.73%; 0.59% and 0.79%; and 0.59% and 1.25%. The stock diet fed to some animals consisted of whole oats, mixed legume hay, fresh forage and iodized salt. Water was available to all animals. More than usual care was taken in shifting the guinea pigs from a stock diet to the experimental diets. In most cases a period of three weeks was taken for a complete change of diets, including the force feeding of those animals that were reluctant to change.

The supplements fed as sources of the "antistiffness factor" included crude sugar cane juice,<sup>1</sup> sugar cane bagasse,<sup>2</sup> and dried penicillin mold pad.<sup>3</sup> The cane juice was fed at a level of 0.25 ml per pig per day; the bagasse ad libitum (about 15 gm per day); and the penicillin mold pad incorporated in the diet at the level of 5%.

The wrists were tested for stiffness according to the technique described by van Wagtendonk and Wulzen ('46). All testing was done by one of the present authors (S.E.S.) and every attempt was made to make the observations as objective as possible. It soon became apparent to us that the subjectiveness of this method, along with the variability encountered within guinea pigs, did not justify the relatively precise scale of 1-4 used by other workers. Accordingly, a more conservative scale of judging the degree of stiffness was used, as

<sup>1</sup> Courtesy of Dr. A. G. Keller, Louisiana State University.

<sup>2</sup> See footnote 1.

<sup>3</sup> Courtesy of Dr. J. J. Oleson, Lederle Laboratories.

follows: 0 indicates normal flexing of the wrists; 1 signifies definite stiffness, and 2 denotes a wrist joint that is practically immobile.

Numerous X-ray pictures were taken of stiff and normal wrist joints.

### RESULTS

The treatments used and some of the results are summarized in table 1.

In the first experiment routine testing of the wrists was started, but the high rate of mortality indicated the unsatisfactory nature of the Oregon diets and the testing was abandoned. Mortality was particularly high (96%) in those animals fed the skim milk diet with or without the supplement

TABLE 1  
*Summary of treatments and results*

DIET	GUINEA PIGS					MORTALITY
	No used	Av. initial wt.	No. stiff			
			Con- sist ent	At times	Neg- ative	
	<i>gm</i>					
	<i>First experiment</i>					
Skim milk	23	335				22 in 2-3 wks.
Skim milk + cane juice	15	321				15 in 2-3 wks.
Purified	15	360				10 in 2-25 wks.
Purified + cane juice	15	332				10 in 2-28 wks.
Dry skim milk	15	302				10 in 3-7 wks.
Dry skim milk + cane juice	15	286				10 in 1-12 wks.
Stock	22	292				2 in 4 wks.
	<i>Second experiment</i>					
Lederle	15	460	6	7	2	0 in 36 wks.
Lederle + mold pad	15	473	10	3	2	5 in 11-36 wks.
Purified	17	410	2	4	11	15 in 2-18 wks.
Purified + mold pad	8	452	0	1	7	8 in 2-17 wks.
Purified + bagasse	9	454	1	1	7	6 in 2-20 wks.
Calf meal	5	519	3	1	1	2 in 15 wks.
Calf meal + mold pad	5	523	1	2	2	1 in 3 wks.
Calf meal + bagasse	5	475	2	1	2	2 in 3-7 wks.
Dry skim milk	12	500	0	3	9	12 in 2-10 wks.
Dry skim milk + cane juice	5	473	2	0	3	4 in 3-4 wks.

of cane juice. Those guinea pigs fed the dry skim milk diet with or without cane juice fared little better (66% mortality). Although mortality was also high in those fed the purified diet, the guinea pigs lived for a longer period of time.

In the meantime our attention had been directed to a diet of natural feedstuffs used successfully to develop a wrist stiffness in the Lederle Laboratories and plans were made to study it, as well as a commercial calf meal enriched in phosphorus which in the past had been shown in this laboratory to produce extensive soft tissue calcification in the rat. Also, additional groups were fed the purified and the dry skim milk diets used by the Oregon workers supplemented with other reported sources of the "antistiffness factor."

In the second experiment, summarized in table 1, it will be noted that those animals fed the dry skim milk diet again suffered a high mortality (100%), even when the ration was supplemented with cane juice. Mortality in those fed the purified diet was also high even when supplements of penicillin mold pad or sugar cane bagasse were fed. On the contrary, mortality was low in those animals fed either the Lederle or the calf meal diets.

In the second experiment the wrists of all animals were routinely tested over a period of 40 weeks. In agreement with the experiences of others, some guinea pigs (19%) had stiff wrists when received from the dealer. It is noted in table 1 that a high percentage of those fed the Lederle diet and the calf meal developed stiff wrists. The table designates the number which were consistently stiff over a period of 40 weeks, as well as those which varied from time to time. This variation in our animals was large even with the conservative scale which we used. This observation is in disagreement with Petering et al. ('48), who stated that "no spontaneous cures were observed in our animals," but is in agreement with Oleson et al., who observed about 13% of spontaneous cures. As an example of the variation which we encountered, table 2 presents periodic observations of the stiffness of the wrists of the 15 guinea pigs fed the Lederle basal diet. Our data give no

evidence that either penicillin mold pad or sugar cane bagasse significantly affects the incidence of wrist stiffness. Some stiff wrists were observed in the guinea pigs fed the purified diet, but the incidence was not nearly so high as in those fed either the Lederle or the calf meal diets. The guinea pigs fed the dry skim milk diet did not survive long enough to permit a valid test of their wrists.

TABLE 2

*Variability of wrist stiffness in guinea pigs over a period of 40 weeks*

GUINEA PIG <sup>1</sup> NO.	VARIABILITY														
1	0	1	0	0	0	0	0	0	0	0	0	0	1	1	0
2	0	0	1	1	1	1	0	0	0	0	0	0	0	0	0
3	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
4	0	0	1	1	1	0	1	1	1	1	1	1	0	0	0
5	0	1	2	2	1	1	2	2	2	1	1	1	0	1	1
6	2	1	1	1	2	1	2	2	2	2	2	2	1	2	2
7	0	0	0	0	1	0	1	1	2	0	0	0	0	1	0
8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
9	1	1	1	1	1	1	2	1	1	1	1	2	2	2	2
10	1	2	1	2	1	2	1	1	1	2	2	2	1	1	2
11	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
12	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1
13	1	1	1	1	1	0	0	1	1	1	0	1	0	0	0
14	0	0	0	1	1	1	0	1	0	1	0	0	0	0	0
15	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0

<sup>1</sup> Right wrist only.

Toward the end of the experiment 6 pairs of guinea pigs were carefully chosen to test the curative effects of ergostanyl acetate.<sup>4</sup> These animals were paired on the basis of previous treatment and degree of stiffness. They all were judged definitely stiff. One member of each pair was then fed 25  $\mu$ g daily of ergostanyl acetate, 5 times the effective dose reported by Oleson et al. Wrists were tested at 5-day intervals. No difference was observed between the two groups either during the feeding of this supplement or at the end of 4 weeks. Petering et al. ('48) also found ergostanyl acetate ineffective at the

<sup>4</sup> Furnished by the Lederle Laboratories.

25  $\mu$ g per day level, but did report a favorable response at a level of 100  $\mu$ g per day.

X-ray pictures of the wrist joints of 16 stiff guinea pigs were compared with X-ray pictures of normal wrists in those guinea pigs fed the same diets, as well as with stock animals, and no evidence of any abnormality was noted.

The rates of growth are of secondary importance in this study and detailed data concerning them are not presented. The guinea pigs fed the skim milk or the dry skim milk diets, with or without cane juice, for the most part lost weight steadily from the beginning. Guinea pigs fed the purified diet with or without the various supplements did not grow but practically maintained their original weight until a short time before death. Those fed the Lederle or the calf meal diets made good weight gains throughout the experiment. The addition of the mold pad appeared to decrease food consumption and guinea pigs fed such rations made slower rates of gain than did other comparable animals.

It was previously mentioned that one of the symptoms described by the Oregon group was a calcification of the soft tissues. We have likewise observed this lesion, particularly in those fed the purified diet for a period of months. In these guinea pigs there appeared to be no correlation between the degree of tissue calcification and the wrist stiffness. Petering et al. ('48), using a modification of the Oregon purified diet (no irradiated yeast or cod liver oil), did not observe tissue calcification up to 4 to 6 months, even though the guinea pigs were very stiff. There is thus a suggestion that the wrist stiffness and the tissue calcification are different syndromes. This phase of the problem is being studied further and will be reported later.

#### DISCUSSION

To present a reasonable interpretation of the present position of the wrist stiffness syndrome in guinea pigs is difficult. It appears to be established, however, that guinea pigs will develop a stiffness of this joint when fed a rather wide variety of diets. In this aspect of the problem the Oregon workers

have been supported by the studies here described, although it is still difficult to see how this group could have published so many apparently clear-cut results when using diets that have in other laboratories led to such large weight losses and high rates of mortality. It is, of course, possible that there is a marked genetic difference in the animals used by the Oregon group and those used by the rest of us, but it is not probable that this would account for differences of such magnitude in the results obtained.

Confusion as to the identity of the "antistiffness factor" is even greater. A recent paper of Simonsen and van Wagendonk ('47) indicating a protective factor effective at a level in the order of  $0.00002 \mu\text{g}$  per day will not be accepted by many without more rigid proof. While some of the steroids studied by Oleson et al. ('47) may have antistiffness activity, the results reported here as well as those obtained by Petering et al. ('48) indicate a much lower potency than that published by Oleson and associates for the most active steroid—ergostanyl acetate. Petering et al. reported a favorable effect when  $100 \mu\text{g}$  daily doses of ergostanyl acetate were fed. This interpretation was based on a change in average stiffness rating from 3.0 to 3.7 in 15 days, which was considered significant. A mean difference of 0.7 unit is indeed a small change to be considered significant, even when that change is objectively measured, as the wrist stiffness was not. The most recent paper from the Oregon workers (Christensen et al., '48) concluded that "the (wrist stiffness) assay procedure is not suitable for quantitative analytical purposes." In these published data mean differences within a treatment group differed as widely as 1.8 stiffness units. Evidence that sugar cane juice or bagasse or penicillin mold pad are rich in the antistiffness factor could not be confirmed in our studies.

The Oregon publications discuss the wrist stiffness and the tissue calcifications as if they were manifestations of the same deficiency syndrome. The preliminary observations reported here and the observations of Petering et al. suggest that they may be two different syndromes. The latter workers did not

observe calcification at all, even though severe stiffness was noted. Their basal diet differed from the Oregon purified diet in not containing irradiated yeast or cod liver oil. Whether or not the high level of vitamin D furnished by a diet containing 10% of irradiated yeast will lead to toxic calcification in the guinea pig is unknown. However, this is a possibility, since rough estimates show that such guinea pigs received approximately 22,000 I.U. of vitamin D for each 25 gm of diet consumed.

#### SUMMARY

The occurrence of stiff wrist joints and tissue calcifications in guinea pigs fed certain diets has been confirmed. In our hands the diets originally used by the Oregon workers have proved unsatisfactory for this purpose, and our evidence does not support the view that the joint stiffness and the tissue calcifications are a part of the same syndrome. Evidence was not obtained that sugar cane juice, sugar cane bagasse, penicillin mold pad or ergostanyl acetate possess antistiffness activity.

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# OVULATION, FERTILIZATION, AND TRANSPORT OF OVA IN OLD, VITAMIN E DEFICIENT RATS<sup>1</sup>

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The number of pregnancies in rats of widely different ages fed vitamin E deficient diets was found to be significantly lower than in controls raised on the same diet supplemented with alpha-tocopherol acetate (Kaunitz and Slanetz, '47). When single doses of alpha-tocopherol acetate were given to deficient, old females after mating, the number of pregnancies increased significantly (Kaunitz and Slanetz, '48). It was concluded that the failure of deficient females to become pregnant was due to disturbances of the implantation process rather than to failure of ovulation, fertilization or transport of the ova.

In order to test the validity of this conclusion and to obtain information on the ovarian function in vitamin E deficiency, it was decided to study the general reproductive phenomena in old, vitamin E deficient rats. The experiments were to include observations on the character of the female mating behavior, the number of ova ovulated and fertilized, and the rate of movement of the ova through the oviducts. Females of comparable ages from the same colony and maintained on the

<sup>1</sup> Aided by a grant from the Williams-Waterman fund of the Research Corporation.

same diet, but supplemented with alpha-tocopherol, served as controls.

### MATERIALS AND METHODS

Twenty experimental and 22 control albino rats derived from a highly inbred colony ranging in age from 149 to 486 days were used. They represented the three to 5 generations maintained continuously on the semi-synthetic diet shown in table 1.

TABLE 1  
*Composition of vitamin E deficient diet*

BASAL MIXTURE		SUPPLEMENTS TO BASAL MIXTURE	
	%		mg/kg
Casein, crude	30	Thiamine chloride	2
Cerelose	54	Riboflavin	4
Lard, commercial	10	Pyridoxine	4
Salt mixture <sup>1</sup>	4	Calcium pantothenate	10
Celluration	2	p-Aminobenzoic acid	300
		Choline	1000
		Inositol	1000
		Vitamin K	4
		Oleum percomorphum	200

<sup>1</sup> Hawk, Oser and Summerson ('47).

Since lard was the only measurable source of tocopherol in the diet it was estimated that the daily intake of tocopherol ranged between 20 to 40  $\mu$ g.<sup>2</sup> This diet had previously proved satisfactory in studies of growth and bioassays (Kaunitz, '46; Kaunitz and Beaver, '46). The control animals were kept on the same diet supplemented by 3 mg of synthetic dl-alpha-tocopherol acetate <sup>3</sup> per 100 gm diet, allowing a daily intake of about 300  $\mu$ g.

When the region of the iliac crest of a female rat in heat is manually stroked, or when she is mounted by a vigorous

<sup>2</sup> We are indebted to Dr. Philip L. Harris and Dr. Mary L. Quaife of Distillation Products, Inc., Rochester, New York, for their help in the assay of lard samples.

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male, the characteristic posture of lordosis is assumed. This posture is characterized by extension of the limbs, arching of the back and raising of the pudendal area and tail. The onset of heat can be accurately determined by this response. The relationship between the onset of heat and ovulation is sufficiently definite that the latter may be said to occur approximately 10 hours after the beginning of heat (Blandau, Boling and Young, '41; Boling, Blandau, Soderwall and Young, '41).

Females in heat were placed with vigorous males in order to observe the character of the heat response and to determine the time of insemination. At varying intervals after mating the females were killed and their reproductive tracts examined to ascertain the position of the ova, the total number ovulated and fertilized, and whether the fertilized ova were developing in a normal manner.

#### EXPERIMENTS

##### *Observations on vitamin E deficient rats*

The general character of the pro-estrous and estrous behavior of these females was similar to that of the controls. The onset of behavioral estrus in the older animals of both the experimental and control groups was neither as abrupt nor as intense as in younger females. In the majority of animals a definite lordosis response could be elicited either by manual manipulation or as the result of mountings of vigorous males (table 2). Four of the animals in which the behavioral response was not as vigorous eventually mated and fertilized ova were found later in their reproductive tracts. One of the females showed a typical male-like behavior characterized by frequent mounting and considerable genital exploration of other females and males. When this female was subsequently killed ovulation had not occurred; the ovaries, however, contained many large, possibly cystic follicles. One of the 20 females in this group died during the period of observation. Autopsy revealed that both oviducts and cornua contained large amounts of purulent material.

TABLE 2

*Mating behavior, number of ova ovulated and fertilized, and their position in the reproductive tract of old vitamin E deficient rats*

ANIMAL NUMBER	AGE	CHARACTER OF MATING BEHAVIOR	HOURS AFTER MATING AND MALE KILLED	LOCATION OF OVA	TOTAL NO. OVA RECOVERED	NUMBER OF NORMAL OVA	NUMBER OF ABNORMAL OVA	REMARKS
RL-12	243	Vigorous	13	"Tubal sac"	6	6	0	all fertilized
L-25	269	Vigorous	15	"Tubal sac"	9	9	0	all fertilized
L-29	242	Weak	15	"Tubal sac"	7	6	1?	{ 1 fertilization one ovum
RL-4	149	Weak	24	Upper Isthmus	10	10	0	2 cell stage
RL-5	239	Vigorous	24	Upper Isthmus	9	9	0	2 cell stage
F-81	361	Vigorous	24	Upper Isthmus	9	8	1	8 normal ova
J-2	427	Vigorous	36	Upper Isthmus	7	7	0	2 cell stage
RL-3	243	Vigorous	36	Upper Isthmus	9	9	0	2 cell stage
L-14	350	Vigorous	48	Middle Isthmus	7	7	0	2 cell stage
FJ-31	399	Vigorous	48	Middle Isthmus	9	9	0	all fertilized
L-6	352	Vigorous	60	Distal Isthmus	8	6	2	{ 2 fragmenting ova 5-4 cell; 1-3 cell
ST-2	352	Vigorous	96	Lower Isthmus	8	8	0	morula stage
F-12	306	Vigorous	96	Cornua	10	10	0	blastocysts
L-26	244	Vigorous	96	Cornua	8	5	3	{ morula stage 3 fragmenting
F-39	374	Weak	120	Cornua	3	0	3	{ 3 fragmenting ova
J-10	404	Vigorous	120	Cornua	5	4	1	{ 4 blastocysts 1 fragmenting
L-21	262	Weak	120	Cornua	7	5	2	{ 5 blastocysts 2 fragmenting ova
L-13	381	Vigorous	192	....	0	0	0	{ both cornua filled with bloody fluid
Total					131	118	13	
average					7.2	6.5	0.7	
range					0-10	0-10	0-3	

A total of 131 ova were recovered from 18 females in this group (table 2). The ova generally were located within the segments of the reproductive tract expected for the particular time interval between ovulation and death (Huber, '15). For example, the ova of all animals examined up to 20 hours after ovulation were confined to the dilated loops of the ampulla and were still surrounded by a varying number of layers of granulosa cells. Ova of animals examined 48 or 72 hours after mating were located in the proximal and distal loops of the isthmus, respectively.

The rate of development in 118 (90%) of the 131 ova recovered appeared to be within normal limits. The remaining ova (13) were in various stages of fragmentation. Whether their death was due to failure of fertilization or to abnormal development after fertilization could not be determined.

#### *Observations on control rats*

Table 3 summarizes the findings with respect to the control group, which had been permitted a daily intake of about 300  $\mu$ g alpha-tocopherol acetate. Three of the 22 females in this group died of unknown causes during the observation period. Two additional females failed to come into heat but subsequent examination of their ovaries showed several sets of recent corpora lutea. The general pattern of mating behavior was similar to that of the E deficient group. Two of the three females whose lordosis response was not as vigorous as that of younger, normal females nevertheless mated, and fertilized ova were recovered from their reproductive tracts. Another female, found to be in heat by manual manipulation, refused to mate with a vigorous male. Subsequent examination revealed that ovulation had occurred.

One hundred and sixteen ova were recovered from 17 females, 81% of which were developing normally. Six per cent of the ova recovered were not fertilized. The rate of transport of ova through the oviducts was normal.

TABLE 3  
Mating behavior, number of ova ovulated, and their position in the reproductive tract of old rats raised on a semi-synthetic, "complete" diet

ANIMAL NUMBER	AGE	CHARACTER OF MATING BEHAVIOR	HOURS AFTER MATING ANT-MAL KILLED	LOCATION OF OVA	TOTAL NO. OF OVA RECOVERED	NUMBER OF NORMAL OVA	NUMBER OF ABNORMAL OVA	REMARKS
<i>data</i>								
II-87	393	Vigorous	15	"Tubal sac"	5	5	0	
D-80	405	Vigorous	20	"Tubal sac"	8	7	1	1 unfertilized
M-88	272	Vigorous	48	Middle Isthmus	5	0	5	not fertilized
KD-57	174	Weak	48	Middle Isthmus	7	7	0	2 cell
KD-46	361	Vigorous	48	Middle Isthmus	8	8	0	7-2 cell; 1-4 cell
KM-22	281	Vigorous	48	Middle Isthmus	8	8	0	2 cell stage
KK-18	271	Vigorous	48	Middle Isthmus	9	9	0	2 cell stage
KM-11	407	Weak	48	Middle Isthmus	10	4	6	4-2 cell; not fertilized
DII-27	373	Vigorous	60	Cornua	6	6	0	blastocysts
KK-35	272	Weak	72	Lower Isthmus	6	0	6	unfertilized
II-90	402	Vigorous	72	Lower Isthmus	8	7	1	7-2 cell; 1 not fertilized
D-82	403	Vigorous	96	Cornua	3	3	0	Normal implantation
KM-63	400	Vigorous	184	Cornua	8	8	0	Normal implantation
KK-45	278	Vigorous	184	Cornua	8	8	0	Normal implantation
K-23	486	Vigorous	192	Cornua	5	5	0	Normal implantation
K-19	377	Vigorous	192	Cornua	5	5	0	Normal implantation
DII-26	382	Vigorous	288	Cornua	7	4	3	3 empty implantation cavities
Total					116	94	22	
average					6.8	5.5	1.1	
range					3-10	0-9	0-6	

## DISCUSSION

One of the most striking aspects of vitamin E deficiency in the rat is the testicular degeneration resulting in cessation of sperm production (Mason, '33). No comparable effect on the ovaries of rats has been observed (Stähler, Hebestreit and Fladung, '40). Ovulation and fertilization as well as the development of ova during their sojourn in the oviduct were shown in the present experiments to be normal even in old, deficient animals.

Wiesner and Bacharach ('37) reported that prolonged vitamin E deficiency reduces the intensity of reproductive behavior in male rats reared on an E deficient diet from the time of weaning. Similarly, Martin and Moore ('39) found that female rats deprived of vitamin E for prolonged periods showed abnormal estrous cycles. Although the length of the reproductive cycles was not determined in either group of animals in the present investigation, one obtained the impression that the cycles in both groups were considerably longer than in younger animals. However, when a female came into heat her behavioral response was of sufficient vigor to allow successful mating and insemination. The general decrease in the intensity of the behavioral response in both groups of animals is interpreted as an effect of aging, since a similar diminution of sexual behavior is commonly seen in older breeding animals.

The reduced ability of the vitamin E deficient rat to become pregnant appears to be caused by uterine rather than ovarian dysfunction. Intense pigmentation of the uterine smooth muscle is characteristic of advancing age in E deficient animals, yet results to date indicate that this does not seriously impair the physiological response of this organ to sympatho- and parasympathomimetic drugs. The possibility nevertheless exists that metabolic changes related to this phenomenon could impair the mechanism of implantation or early placental development (Martin and Moore, '39).



## SUMMARY

In older vitamin E deficient female rats the pattern of mating behavior, number of ova ovulated and fertilized, and early development and transport of ova through the oviducts is comparable to that of females raised on the same diet supplemented by alpha-tocopherol. The low fecundity rate of vitamin E deficient rats is, therefore, apparently due to disturbances of the uterine implantation of the ovum rather than to failure of ovulation, fertilization, or transport of ova through the oviducts.

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# THE UTILIZATION OF LACTOSE BY THE MATURE FOWL<sup>1</sup>

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Guba ('31) reported that rats rapidly declined in weight and died when galactose was used as the carbohydrate in a fat-free diet. Bilateral cataract was observed in rats fed 70% lactose by Mitchell and Dodge ('35). Schantz et al. ('38) fed rats on mineralized skim milk and found sugar in the urine, which was identified as galactose; similar animals fed mineralized whole milk made efficient utilization of the milk sugar. These workers noted that 3 to 4% fat in the mineralized skim milk diet prevented the loss of sugar in the urine. In a further study Schantz and Krewson ('39) reported that even chain fatty acids with over 12 carbon atoms fed at a rate of 3 to 4% were effective in preventing the loss of galactose in the urine of rats fed mineralized skim milk, while such fatty acids of less than 12 atoms were not effective in preventing this loss.

It was reported by Couch et al. ('48) that the feeding of 15% lactose in a purified diet to laying hens appeared to have a deleterious effect on egg production after about three weeks. Twenty-five per cent dried whey (equivalent to 15% lactose) had a similar effect. It was noted further that neither

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lactose nor dried whey promoted the intestinal synthesis of biotin.

The present investigation was initiated to study the utilization of lactose by the laying hen. It was deemed advisable to use 63% lactose since it was thought that the use of this sugar as the only carbohydrate in a purified diet would give more accurate data on its utilization by the laying hen.

#### MATERIALS AND METHOD

Single-comb White Leghorn pullets reared on the Experiment Station range were placed in individual laying cages with raised screen bottoms. All birds were artificially inseminated weekly with mixed semen from New Hampshire cockerels. All eggs laid were marked with the hen's number and date. Settings were made weekly. The eggs were candled on the 7th and 17th days of incubation, at which times the infertile eggs and dead embryos were removed. All eggs which were removed on candling, and also those which failed to hatch, were broken and the age of the embryo at the time of death was estimated.

The composition of the experimental diets is given in table 1. Five experiments were conducted. Four birds were included in all groups. In experiment 1 diets B36 and B37 were fed for a 4-week period. Experiment 2 included diets B36A and B41-44A. At the end of the first two weeks diets B43 and B44 were discontinued and two hens from each of these groups were fed diet B44A for the remainder of the experiment. Diet B34 served as a control diet for this 7-week experiment. In experiment 3 diets B36A, B44A and B50 were fed for a 5-week period. B34 again served as the positive control. The birds were continued on diet B36A and 44A for the metabolism studies outlined below after experiment 3 was terminated.

Two metabolism experiments were carried out. In the first case 4 birds were fed the practical all-mash diet (Diet B1 of Robblee et al., '48) ad libitum and in addition two of these birds were given by capsule 25 gm of a 1:1 mixture of galac-

tose and glucose, while the remaining two were given 25 gm of lactose. Blood was obtained by puncturing the wing vein. Blood galactose determinations were made on individual hens each hour for 4 hours after the administration of the sugar. In the second metabolism experiment one group of 4 hens

TABLE 1  
*Composition of experimental diets (parts by weight)*

COMPONENTS	D I E T									
	B34	B36	B36A	B37	B41	B42	B43	B44	B44A	B50
<i>Dietary components kept constant</i>										
Casein										
purified	18	18	18	18	18	18	18	18	18	18
Gelatin	5	5	5	5	5	5	5	5	5	5
Salts IV	5	5	5	5	5	5	5	5	5	5
Liver										
fraction L	4	4	4	4	4	4	4	4	4	4
Fish oil										
(3000 A, 400 D)	2	2	2	2	2	2	2	2	2	2
Choline	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Crystalline vitamins <sup>1</sup>										
Oyster shells — ad libitum										
<i>Dietary components that were varied</i>										
Sucrose	63									
Whole milk powder										100 <sup>2</sup>
Lactose		63	63	63	60	58	63	53	38	
Soybean oil	3	3	3		3	3	3	13	28	
Butter oil				3						
Fish solubles					3					
Whole liver powder						5				
Desiccated thyroid							0.125			
Biotin (mg/kg)	0.2		0.2		0.2	0.2	0.2	0.2	0.2	

<sup>1</sup> Crystalline vitamins, in milligrams per kilogram of ration, added to all diets as follows: Thiamine-HCl 4; riboflavin 6; Ca-pantothenate 15; niacin 100; menadione 0.5; pyridoxine-HCl 4; alpha-tocopherol 4.

<sup>2</sup> Fortified haliver oil (60,000 A, 10,000 D) two drops per hen daily. Also, in milligrams per kilogram of ration: Iron 20, copper 2, manganese 50, and iodine 1.

was fed diet B36A and a second group of 4 was fed diet B44A ad libitum. Two birds from each group were given individual doses of 25 gm of a 1:1 mixture of galactose and glucose, while the other two birds were each given 25 gm of lactose by capsule. Individual blood galactose determinations were made at the end of the first, second, third, 6th and 8th hours.

Blood glucose or total blood sugar was determined according to the method of Folin and Malmros ('29) as modified by Horvath and Knehr ('41). In each instance a separate sample (0.1 ml) of blood was collected for the galactose determination, which was carried out as outlined below. One-tenth milliliter of whole blood was pipetted into 10 ml of sodium tungstate-sulfuric acid solution (Folin and Malmros, '29). After centrifuging, 8 ml of supernatant liquid was pipetted into another centrifuge tube and 0.2 ml of fresh live yeast (prepared by washing 6 to 8 times with water—1 gm washed yeast made up to 2 ml) added. After incubation at 37°C. for 30 min., an aliquot (4 ml) was taken and was carried through the procedure outlined above for determining total blood sugar. It was found that the yeast failed to remove completely the reducing substances of the blood from the blood of hens fed sucrose (diet B34). Thus it was necessary to use blood from hens fed sucrose as a blank in each series. In this case the milligram per cent of reducing substances in the blood of hens fed diet B34 was calculated in each determination after fermentation with yeast; this value was subtracted from the milligram per cent galactose observed after fermentation of blood from hens fed lactose, or the blood of those fed the 1:1 mixture of galactose and glucose. The figure thus obtained was termed "milligram per cent galactose."

#### EXPERIMENTAL RESULTS

Experiment 1 was designed to test the effect of butterfat and soybean oil on the intestinal synthesis of biotin with lactose as the carbohydrate. As is shown in table 2, neither diet B36 nor B37 supported egg production. Thus this experiment yielded no data on the relation of fat to intestinal synthesis,

but its results are included here to show the effect on egg production of diets containing lactose as the sole carbohydrate.

The egg production and hatchability data obtained in experiments 2 and 3 are presented in table 2. It may be seen that the rate of production of the birds fed diet B36A fell rapidly. These birds ceased to lay after the second week of the experiment. The addition of fish solubles (B41) or des-

TABLE 2  
*Effect of diets on egg production and hatchability*

EXPERIMENT NO.	DIET NO.	EGG PRODUCTION								HATCHABILITY
		Pre-exptl.	1 week	2 week	3 week	4 week	5 week	6 week	7 week	
			%	%	%	%	%	%	%	%
1	B36	—	46	0	0	0				80
	B37	—	46	0	0	0				67
2	B34	79	79	68	70	79	79	64	71	90
	B36A	75	43	6	0	0	0	0	4	100
	B41	75	43	21	4	4	0	0	4	89
	B42	68	35	10	10	18	35	21	14	75
	B43	75	39	0 <sup>1</sup>	—	—	—	—	—	100
	B44	61	21	0 <sup>1</sup>	—	—	—	—	—	76
	B44A			<sup>2</sup>	0	26	57	53	43	90
3	B34	68	71	58	64	64	57			70
	B36A	85	47	10	3	0	0			100
	B44A	85	48	57	64	64	57			84
	B50	75	39	18	39	53	43			91

<sup>1</sup> Discontinued.

<sup>2</sup> Two hens from diet B43 and two from B44 given diet B44A.

iccated thyroid (B43), or an increase in the level of soybean oil to 13% (B44) failed to result in improved egg production. The addition of 5% whole liver powder to diet B36 (B42) resulted in some improvement in production, although it is obvious that production was significantly below that obtained with the control diet, B34.

It has been mentioned earlier that the groups fed diets B43 and B44 were discontinued at the end of the second week: the per cent egg production of these groups was zero at this time.

Two hens were selected from each of those groups at the end of the second week and were fed diet B44A (lactose with 30% fat) from the beginning of the third through the 7th week of the test. The egg production of hens fed diet B44A increased, and approached that of those fed B34. From these results it is apparent that 30% fat with lactose (diet B44A) tended to correct the deleterious effect of lactose alone on egg production when fed to the laying hen.

Further evidence that a high level of fat counteracts the deleterious effect of lactose on laying hens is shown in the results of experiment 3. Although production was somewhat erratic, due to the small number of birds in each group, it is obvious that diet B44A supported production at a rate equal to that of the control diet, B34. Egg production of the birds fed dried whole milk (B50) decreased during the first two weeks of the experiment, then increased to a level somewhat below that obtained with the control ration. The whole milk powder was quite hygroscopic and tended to gum up in the beak as it was eaten. One hen of the group refused to eat an appreciable quantity of the diet and laid no eggs after the first week of the experiment. The weight of this bird decreased approximately two-thirds during the experiment, whereas the other hens fed the whole milk powder gained from 200 to 400 gm each during the test. The body weights of hens fed diet B36A fluctuated within about 100 gm of the starting weight in each instance.

The hatchability data for all groups of birds, given in table 2, show that there is no significant difference in this respect in any of the rations fed in these experiments.

The results of the metabolism studies are given in table 3. These data indicate that the presence of 30% fat in a lactose diet aids in the utilization of this carbohydrate. This is apparent when the figure for milligram per cent of galactose in the blood of hens fed the practical diet is compared with that of the hens fed diet 44A, where both groups had received a 25 gm dose of the 1:1 mixture of glucose and galactose. The practical diet contained 5 to 6% fat and when

hens were fed this diet and given 25 gm of the sugar mixture, the blood galactose level was higher throughout the 4-hour period than that of hens fed a lactose diet containing 30% fat. Similar and more convincing results were obtained when the glucose-galactose mixture was fed to one group of hens receiving a ration containing 5% fat (B36A) and to another receiving a diet containing 30% fat (B44A).

TABLE 3

*Effect of fat on blood galactose level after administration of 25 gm of lactose or 25 gm of a 1:1 mixture of glucose plus galactose*

HEN DIET	SUGARS FED	MG % GALACTOSE AT HOURS AFTER FEEDING							
		1	2	3	4	5	6	7	8
Practical	{ Lactose	0	20	7	20				
	{ Glucose + galactose	80	150	148	212				
B44A (30% fat)	{ Lactose	36	18	18	13				
	{ Glucose + galactose	62	91	140	165				
B36A (5% fat)	{ Lactose	0	18	9				8	8
	{ Glucose + galactose	76	124	170				193	191
B44A (30% fat)	{ Lactose	0	30	5				5	5
	{ Glucose + galactose	42	60	82				64	42

In view of the fact that the feeding of lactose in a purified diet (B36A) stopped the hens from laying in such a short time, it was thought that galactose in the blood of the fowl might in some manner be interfering with the secretion of the hormones of the anterior pituitary, which control egg production. However, when hens were fed diet B36A and injected in one case with a preparation of the luteinizing hormone and in another with a preparation of the anterior pituitary, egg production of birds receiving the injections was about the same as that of those fed diet B36A without injections. It was noted that injection of the hormones caused a decided decrease in weight (500 to 600 gm per bird in two weeks).



## DISCUSSION

The egg production data show clearly that the presence of fat in the diet enables the hen to use high levels of lactose, as is indicated by continued egg production.

The effect of fat could be considered as a mere replacement of lactose in the diet. If such is the case, then the addition of sucrose to a lactose diet should have a similar effect, but previous work with a diet containing 15% lactose (Couch et al., '48-diet B33) showed that sucrose in amounts isocaloric to the fat in the present experiments did not prevent this deleterious effect of lactose on egg production.

It was mentioned above that Schantz et al. ('38) observed galactosuria in rats fed skim milk, but when the skim milk was supplemented by 3 to 4% fat the loss of sugar in the urine was prevented. Geyer et al. ('45), also using galactosuria as a criterion, concluded that fat increases the utilization of galactose. Nieft and Deuel ('47) found that the rate of intestinal absorption of galactose in the rat varies inversely with the percentage of fat in the diet. They suggest that the galactosuria may be a "flooding effect" produced by the faster absorption rate of galactose, and that the slowing of this absorption by the fat enables the rat to metabolize the galactose more completely, thus preventing galactosuria.

While our short period blood sugar studies do not indicate an effect of fat on the galactose level of the blood when lactose is fed, the data obtained from the hens fed the mixture of glucose and galactose show that the presence of high fat in the diet results in lower blood galactose levels. This effect might be due, as suggested by Nieft and Deuel, to a slower absorption rate and the prevention of "flooding." It may also be a metabolic effect produced after the galactose reaches the blood. Further work is being carried out in our laboratory to study this possibility.

## SUMMARY

Laying hens fed a purified diet with lactose as the carbohydrate ceased laying in one to two weeks after being placed

on the diet. Thirty per cent fat in the diet corrected the deleterious effect of lactose on egg production. High levels of lactose appeared not to affect hatchability.

In metabolism studies high levels of fat in the diet resulted in lower blood galactose levels when galactose was administered orally.

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# RELATION OF IODINE TO THE GOITROGENIC PROPERTIES OF SOYBEANS<sup>1</sup>

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FOUR FIGURES

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Various investigators have reported that soybeans contain a goitrogenic agent which increases the iodine requirement of chicks and rats. McCarrison ('33) reported a high incidence of thyroid enlargement in rats fed a diet containing raw soybeans. His results indicated that raw soybeans produce goiter even when iodine is fed in large amounts. Sharpless et al. ('39) reported that soybean flour contains a positive goitrogenic property that is partially removed or destroyed by fat solvents (ether or acetone) or by steam. Wilgus et al. ('41) also found that the soybean agent was partially inactivated by heat, but they were unable to extract the factor with solvents similar to those employed by Sharpless. Both groups of investigators showed that the addition of small amounts of iodine to the goitrogenic diets will largely prevent the thyroid enlargement. They also showed that the goitrogenic property of soybeans is not due solely to their low iodine content. Wilgus et al. found the iodine content of a ration containing 15% casein to be approximately the same as that in

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the goitrogenic soybean rations, yet the casein ration caused only minor thyroid enlargement in comparison to the soybean rations. Sharpless et al. adjusted the iodine content of a soybean diet by supplementation to a level comparable to that of the stock ration and then measured the thyroid size and iodine content of glands from animals in both experimental and control groups. The thyroid glands of the stock animals were normal but the glands from the soybean rats were enlarged and low in iodine.

The rachitic ration of Steenbock and Black ('25), which was modified by Levine et al. ('33), has been reported by several investigators (Krauss and Monroe, '30; Thompson, '32; Hall, '46) to produce goiter in rats. The composition of this ration, as modified, follows: yellow corn, 76 parts; wheat gluten, 20 parts;  $\text{CaCO}_3$  three parts;  $\text{NaCl}$ , one part; and irradiated yeast, 0.2 part. Levine et al., Thompson, and Hall have shown that the goitrogenic properties of this ration are prevented by small amounts of iodine. In cases where this diet fails to produce goiter, either the ration contains significant amounts of iodine or the rats employed in the experiment have a high initial store of reserve iodine. Levine et al. also showed that 1 to 2  $\mu\text{g}$  of iodine are required per day to maintain normal thyroid structure in the growing rat. The requirement was determined by comparing the weight, iodine content and dry matter content of thyroid glands from rats supplemented with various levels of iodine (KI). Most investigators accept the iodine requirement of the growing rat as determined by these workers as being the normal value. The question of whether this requirement is altered by diverse types of diets has not been adequately investigated. The fact that animal proteins such as casein (Remington, '37) generally contain significant amounts of iodine has prevented their general use in iodine requirement studies.

#### EXPERIMENTAL

The effect of iodine supplements upon the size, iodine content and histological structure of the thyroid gland was

studied with rats fed various types of soybean-containing rations. In the first experiment two groups of rats (6 per group) were fed a soybean ration to test the effect of soybeans in causing increased thyroid enlargement. One of the groups received no added iodine and the other received an iodine supplement of 2.0  $\mu$ g of iodine per rat per day. The soybean ration consisted of 10 parts of raw soybeans added to 90 parts of the basal ration.

### *Rations*

The basal ration selected was similar to the previously described diet employed by Levine et al. ('33). The percentage composition of the ration follows: wheat gluten, 30; ground yellow corn, 68; NaCl, 1; and CaCO<sub>3</sub>, 1. Reagent grade NaCl and CaCO<sub>3</sub> were used in the rations. The water-soluble vitamins were dry mixed with the wheat gluten before the ration was prepared. The vitamin levels added per kilogram of ration follow: 6.0 mg thiamine hydrochloride, 9.0 mg riboflavin, 9.0 mg pyridoxine hydrochloride, 60.0 mg calcium pantothenate, 20.0 mg nicotinic acid, 300.0 mg *p*-aminobenzoic acid, 1.0 gm inositol, 2.0 gm choline chloride, 0.1 mg biotin and 0.3 mg folic acid. The fat-soluble vitamins were dissolved in corn oil<sup>2</sup> and fed by dropper once a week. The amount of each of the fat-soluble vitamins fed per rat per week was: 3.0 mg  $\alpha$ -tocopherol, 10.0  $\mu$ g calciferol, 0.15 mg 2-methyl-1, 4-naphthoquinone and 0.16 mg  $\beta$ -carotene.

Great care was used in the preparation of the experimental rations. The corn and soybeans were ground in a burr mill which had been thoroughly washed previous to use. The rations were mixed in a porcelain jar by stirring with a glass rod.

The conditions employed in the second experiment (table 1, expt. 2) were similar to those of the first except that a salt mixture was substituted for the CaCO<sub>3</sub> and NaCl in the basal ration. A supplementary group (6 rats) which received the

<sup>2</sup> Mazola.

TABLE 1  
*Iodine requirement of rats fed raw and heated soybeans<sup>1</sup>*

EX- PERI- MENT NO.	SUPPLEMENT		TOTAL IODINE INTAKE PER RAT PER DAY	BODY WEIGHTS: INITIAL AND FINAL	THYROID WEIGHTS (FRESH) PER 100 GM BODY WEIGHT			IODINE CONTENT OF THYROID		
	Soybean	Iodine			Average	Range	Average	Range	Total	On dry weight basis
%	$\mu$ g	$\mu$ g	gm	mg	mg	$\mu$ g	$\mu$ g	%		
1	None	None	0.26	42-151	18.5	14.8-20.2	0.6	0.5-0.78	0.009	
	None	0.5	0.76	41-148	10.8	9.0-12.5	2.7	1.4-4.4	0.06	
	None	1.0	1.26	42-146	11.3	9.9-12.2	3.4	2.6-4.1	0.08	
	None	2.0	2.26	43-154	10.2	8.9-11.4	9.0	5.5-13.5	0.18	
	10 raw	None	0.30	42-144	17.1	14.1-19.4	0.7	0.6-0.8	0.01	
	10 raw	2.0	2.30	43-146	11.6	10.3-13.2	9.3	4.8-13.2	0.18	
2	None	None	0.25	43-175	24.1	16.7-36.5	0.18	0.13-0.22	0.0017	
	30 raw	None	0.33	44-155	27.8	23.0-39.6	0.21	0.13-0.28	0.0026	
	30 raw	0.5	0.83	44-150	15.0	12.0-17.5	3.0	2.0-3.6	0.04	
	30 raw	1.0	1.33	44-158	12.8	9.8-17.6	4.6	3.7-6.3	0.07	
	30 raw	2.0	2.33	43-154	12.8	10.4-15.3	8.2	4.2-11.2	0.13	
	30 heated	None	0.36	43-177	25.3	15.1-44.0	0.23	0.19-0.26	0.0025	
	30 heated	0.5	0.86	42-166	14.0	10.3-17.1	2.9	1.5-4.4	0.04	
	30 heated	1.0	1.36	42-176	12.7	10.9-13.9	5.3	3.6-7.0	0.08	
30 heated	2.0	2.36	43-165	10.0	8.6-11.3	7.6	5.9-11.0	0.14		

<sup>1</sup> Figures represent the average of 6 rats.

salt mixture in place of  $\text{CaCO}_3$  and  $\text{NaCl}$  had previously been included in experiment 1. The purpose of this group was to determine whether the addition of a more complete mineral supplement would alter the goitrogenic property of the ration. Since the thyroids of the rats receiving the salt mixture were enlarged to the same extent as those receiving  $\text{CaCO}_3$  and  $\text{NaCl}$ , it was decided to add the salt mixture to the basal ration in experiment 2.

The percentage composition of the basal ration employed in experiment 2 follows: ground yellow corn, 66; wheat gluten, 30; and salts, 4. The percentage composition of the salt mixture prepared from reagent grade chemicals was:  $\text{Ca}_3(\text{PO}_4)_2$ , 48;  $\text{NaCl}$ , 30;  $\text{KCl}$ , 10;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 8.0;  $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ , 3.2;  $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ , 0.6;  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.1; and  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ , 0.1. The  $\text{Ca}_3(\text{PO}_4)_2$  was further purified by a procedure described in an earlier paper (Halverson et al., '45). The water-soluble and fat-soluble vitamins were fed at identical levels and in the same manner as in experiment 1.

The level of soybeans added to the basal ration was increased to 30% since no significant goitrogenic effect had been observed with 10% in experiment 1. The heated soybeans were prepared by autoclaving the raw ground beans in thin layers in open glass dishes for 10 min. at 15 lb. pressure. The soybeans employed in both experiments were the Kingwa variety.

The low iodine diets were supplemented with potassium iodide (aqueous solution) by dropper. Levels of 0.5, 1.0 and 2.0  $\mu\text{g}$  of iodine per rat per day were fed the various groups. Since the daily iodine requirement of the growing rat has been reported to be 1 to 2  $\mu\text{g}$ , these levels of supplementation were thought to be the most desirable in order to study any goitrogenic effects.

### *Experimental animals*

Female albino weanling rats (39 to 49 gm) of the Sprague-Dawley strain were employed and 6 animals per group were



used throughout the studies. The animals were housed in individual wire cages. The double distilled water and the experimental rations were fed in porcelain dishes. The growth rates of the rats were followed by weighing the individual animals at weekly intervals. Food consumption was measured frequently.

The animals were kept on the experimental diets 7 weeks, at which time they were killed by ether anesthesia. The thyroids, together with the parathyroid glands, were removed with the aid of a binocular dissecting microscope and weighed immediately on a torsion balance. This balance weighed to 0.1 mg and little, if any, blood or extraneous tissue was left attached to the thyroid. The thyroids used for histological study were prepared in the usual manner using Bouin's fixative and staining with hematoxylin and eosin. In experiment 2 the thyroids from half of the animals of each group were used for histological examination and the remaining thyroids (three from each group) were dried, weighed and saved for iodine analysis. All of the thyroids of animals used in experiment 1 were dried, weighed and saved for iodine analysis.

### *Iodine determinations*

The Chaney method was selected for the iodine determinations because of its simplicity and dependability. Numerous investigators (Leipert, '33; Trevorrow and Fashena, '35; and Matthews et al., '38) have shown that small amounts of iodine can be quantitatively recovered by distillation from a digestion mixture of chromic and sulfuric acids after the addition of a suitable reducing agent. Because of the extreme sensitivity and specificity of the catalytic action of the iodide ion on the reduction of the ceric ion by arsenious acid, Chaney ('40) and Taurog and Chaikoff ('46) have adapted this reaction to the measurement of the small amounts of iodine present in the distillate mentioned above. The procedure followed for measuring the iodine in the thyroid glands was identical with that described by Taurog and Chaikoff, who

used the Chaney method for the determination of plasma iodine. Recoveries were run with inorganic iodine (KI) to determine the percentage recovery with amounts of iodine at levels comparable to those in the thyroid glands. The recoveries from 0 to 10  $\mu\text{g}$  iodine ranged between 65–100%. Suitable correction factors were employed in the gland analysis to correct for the losses determined by the recoveries.

The iodine analyses of the low-iodine rations required an initial combustion. The method of Karns ('32), as modified by von Kolnitz and Remington ('33), was employed. After combustion of the sample and collection of the absorbing liquid and rinsings, the analyses were performed by the same procedure as that used for the iodine in the glands. The recoveries, which were run by burning samples of ration containing added potassium iodide (0 to 4  $\mu\text{g}$ ), averaged 102.8% (81 to 121).

The method was found to be readily applicable to the estimation of thyroid iodine. However, it was more difficult to reproduce results with the rations (combusted samples). The minerals present in the absorbing liquids often caused a precipitation to occur in the digestion flasks. Sufficient analyses were run on the rations and on the rations plus added iodine to enable the iodine values to be averaged. The principal difficulty encountered in determining the iodine content of the various rations was that the values obtained for those containing the salt mixture were approximately three times as high as those obtained on rations containing  $\text{CaCO}_3$  and  $\text{NaCl}$ . The thyroid enlargement on the two rations on the same experiment was very similar (18.5 and 20.2 mg per 100 gm of body weight). On the basis of thyroid enlargement it was concluded that the two rations were very nearly the same in iodine content, even though the values obtained by chemical analysis were different. The iodine values obtained with the ration containing the salt mixture could not be correct, for no appreciable thyroid enlargement would have been obtained at such a high level of iodine intake.

The individual constituents of the rations employed in experiments 1 and 2 were then analyzed for iodine. The iodine content of the basal ration of experiment 1 was the same when the  $\text{CaCO}_3$  and  $\text{NaCl}$  were removed as when they were present. In the case of the basal ration of experiment 2, the iodine content was the same as that of the basal ration of experiment 1 when the salt mixture was removed. The salt mixture was then analyzed separately and found to give an iodine value of the same magnitude as that calculated from the difference between the basal ration with and without the added salt mixture. It would appear from these observations that the high iodine results with the rations containing the salt mixture were due to contamination by some interfering ion present in the minerals. Several ions such as osmium, mercury and silver have been shown by Sandell and Koltzoff ('37) to interfere with the catalytic effect of iodides on the reduction of the ceric ion by arsenious acid. It is possible that traces of an interfering ion may be present in commercial mineral preparations. It is also possible that interfering substances may exist in natural feedstuffs in amounts sufficient to invalidate the results obtained when they are used; but since the iodine values obtained on the natural feedstuffs were very low and within the range expected, they were assumed to constitute an accurate measure of iodine content. It is felt that the iodine values obtained for the rations used in experiment 2 were approximately correct, even though salts were not added to the rations analyzed.

#### RESULTS

The groups fed rations supplemented with potassium iodide showed no significant differences in food consumption or rate of growth from those which received no iodine supplements. The rats of all groups appeared healthy and normal throughout the experiments.

In experiment 1 (table 1) the effects of iodine supplements upon thyroid weight (fresh weight) and iodine content (dry basis) are shown. Supplementing the basal ration with  $0.5 \mu\text{g}$

trophy and mild hyperplasia (fig. 1). There was little colloid present in these thyroids except in the periphery of the lobe. Foci of beginning necrosis were common in the thyroids from the control animals. Thyroids from the groups receiving 30% of raw or heated soybeans with no iodine supplement showed a more severe epithelial hyperplasia and more numerous foci of necrosis (fig. 2). However, no difference in pathology of the thyroids could be detected between animals receiving raw or heated soybeans. There was a reduction in the severity of the epithelial hypertrophy and hyperplasia when the rations of animals receiving raw and heated soybeans were supplemented with  $0.5 \mu\text{g}$  of iodine per rat per day (fig. 3). The acini were generally small but they did contain a pale staining colloid. Foci of necrosis were still frequent in the thyroids of these groups and many acini were found to contain, in addition to the colloid, several pyknotic nuclei and leukocytes within their lumina. The administration of 1.0 or  $2.0 \mu\text{g}$  of iodine was effective in reducing the thyroid pathology in the animals receiving the soybean diets (fig. 4). Histologically, most of these thyroid acini were normal in size and were filled with a dense colloid. However, significant numbers of leukocytes and pyknotic nuclei were still present within the lumina of these acini, and foci of necrosis were still common. No differences in the severity of the thyroid pathology were observed between the groups receiving raw and heated soybeans at the different levels of iodine supplementation.

#### DISCUSSION

The results of our experiments indicate that small amounts of iodine can effectively prevent thyroid enlargement in rats on soybean-containing rations. The amount of iodine necessary to prevent gross enlargement on the soybean rations was within the range of the accepted requirement of 1 to  $2 \mu\text{g}$  of iodine per rat per day. The thyroid weight, iodine content and histological data show that the addition of raw or heated soybeans to the basal ration used increased the iodine re-

quirement of rats to some extent. The iodine analysis values indicate that the addition of either raw or cooked soybeans to the diet did not prevent or significantly alter the uptake of iodine by the thyroid gland. The average iodine content was greater than the critical level of 0.1% (dry basis) when the basal and the soybean-containing rations were supplemented with  $2.0\text{ }\mu\text{g}$  of iodine per rat per day. Small differences in thyroid weight and iodine content also indicate that a more severe goiter was obtained with 30 than with 10% raw soybeans and with raw than with cooked beans. Since no definite histological differences between the groups fed the raw and the heated soybeans were evident, the significance of the slight differences in iodine content and thyroid size between the raw and the heated soybean rations should be appraised with caution.

When 30% of raw or heated soybeans were added to the basal ration, a more severe epithelial hyperplasia and necrosis of the thyroid gland resulted than when rats were fed the basal ration only. The addition of small amounts of iodine ( $0.5$  to  $2.0\text{ }\mu\text{g}$ ) to the soybean-containing rations resulted in a definite decrease in the severity of the glandular pathology. The colloid accumulation which occurred with iodine supplementation was accompanied by greater iodine content as measured by chemical analysis. Even when the rats receiving the soybean-containing rations were supplemented with  $1.0$  and  $2.0\text{ }\mu\text{g}$  of iodine per day, the gland structure was not completely normal, for areas of necrosis were present. It is not known whether supplementation with amounts of iodine larger than  $2.0\text{ }\mu\text{g}$  would completely prevent this necrosis. Since no significant gland enlargement occurred at levels of  $1$  to  $2\text{ }\mu\text{g}$  of iodine per day, it is concluded that the amount of iodine supplied was adequate to enable the thyroid gland to make a sufficient amount of the iodine-containing hormone (thyroglobulin) to supply the normal physiological requirements of the growing rat.

The results obtained are in essential agreement with the data of Sharpless et al. ('39), who found that autoclaved soy-

beans produced less thyroid enlargement than the raw beans. When they fed rats a raw soybean ration containing various levels of added iodine, they also found that the level of iodine necessary to prevent thyroid enlargement was greater than the normal requirement. They did not show whether a definite decrease in iodine requirement resulted from heating the soybeans. Wilgus et al. ('41) reported that unheated soybeans produce much larger goiters in chicks than heated beans. They also noted that small amounts of iodine are effective in lessening the severity of goiter on the soybean-containing rations, but they did not report sufficient iodine supplementation experiments to determine the extent to which the iodine requirement on such rations is increased above the normal. These workers obtained with chicks greater differences in thyroid weights between raw and heated soybeans than Sharpless and associates did with rats. Wilgus et al. examined histologically thyroids from chicks and rats on the same soybean-containing ration and concluded that the chick thyroid is more sensitive to goitrogenic rations than is that of the rat.

The present authors agree with Wilgus et al. that the slight goitrogenic property of raw and heated soybeans is not of practical importance in animal nutrition, since small amounts of iodine can effectively prevent the resulting thyroid enlargement. A ration containing 1% of iodized salt (0.01% potassium iodide) supplies a rat consuming 10 gm of ration per day with 7.6  $\mu\text{g}$  of iodine. Such an amount is adequate to prevent goiter under all ordinary conditions.

#### SUMMARY

1. The thyroid enlargement obtained with rations containing 10 and 30% of raw or heated soybeans was effectively prevented with 1 to 2  $\mu\text{g}$  of iodine per rat per day. The iodine requirement of rats was slightly greater on soybean-containing rations than on the basal ration employed.

2. The reduced thyroid enlargement resulting from heat treatment indicates that the goitrogenic property of soybeans' is partially heat labile.

3. Since small amounts of iodine effectively correct the mild goitrogenic property of soybeans, no harmful effects should result from the use of soybeans in practical rations.

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## PLATE I

## EXPLANATION OF FIGURES

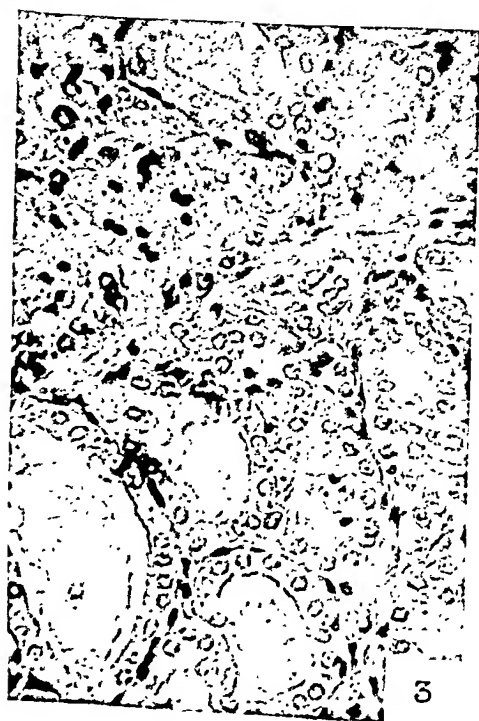
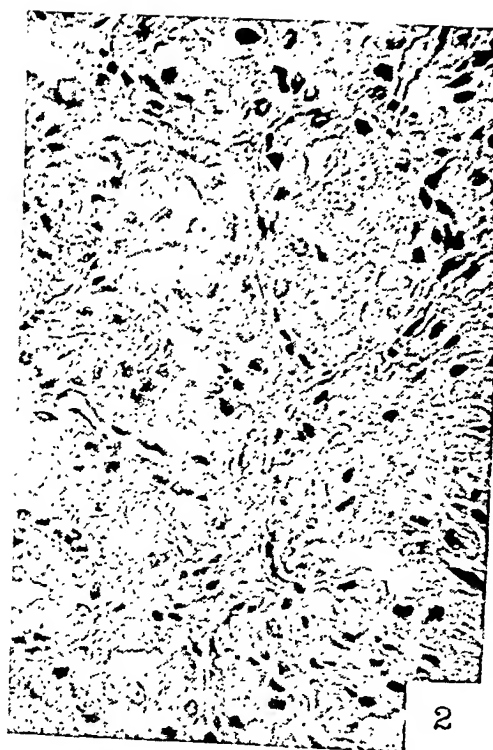
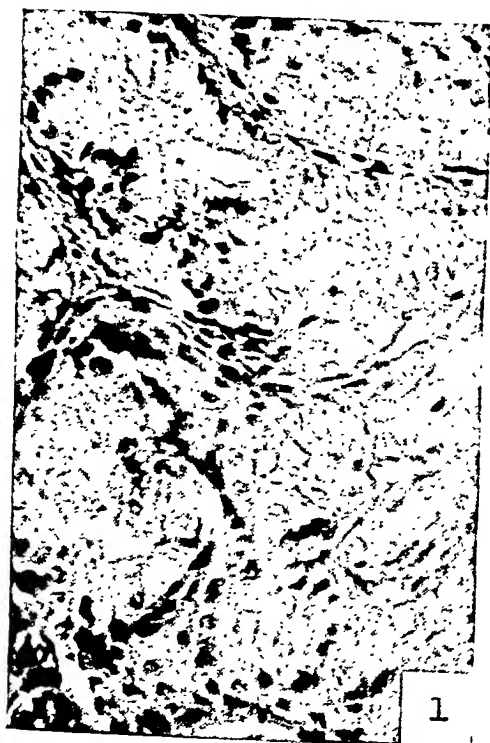
1 A section of the thyroid gland of a rat which received the basal ration only. Epithelial hypertrophy, hyperplasia and necrosis. Hematoxylin and eosin  $\times 350$ .

2 A section of the thyroid gland of a rat which received the basal ration with 30% raw soybeans. Epithelial hypertrophy and more severe necrosis. No difference in the pathology of the thyroids could be found between animals receiving the raw or cooked soybeans. Hematoxylin and eosin  $\times 350$ .

3 A section of the thyroid gland of a rat which received the basal ration containing 30% cooked soybeans plus 0.5  $\mu\text{g}$  of iodine per day. Small acini filled with colloid and mild necrosis. No difference in the pathology of the thyroids could be found between animals receiving the raw or cooked soybeans. Hematoxylin and eosin  $\times 350$ .

4 A section of the thyroid gland of a rat which received the basal ration with 30% cooked soybeans plus 1  $\mu\text{g}$  of iodine per day. A few pyknotic nuclei and lymphocytes within the colloid-filled lumina. Supplementation with 2  $\mu\text{g}$  of iodine per rat per day gave the same histological picture of the thyroid as did supplementation with 1  $\mu\text{g}$  of iodine. Hematoxylin and eosin  $\times 350$ .







# VITAMIN B<sub>6</sub> DEFICIENCY IN THE SYRIAN HAMSTER <sup>1</sup>

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TEN FIGURES

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While vitamin B<sub>6</sub> deficiency has been investigated extensively in the rat and other animal species (i.e., dog, chick, swine, monkey and mouse), there have been few and inconclusive observations made on this deficiency in the Syrian hamster. Routh and Houchin ('42) reported briefly an "acrodynia-like" dermatitis around the mouth, loss of weight and death within 24 days in hamsters fed a pyridoxine-free diet. Cooperman, Waisman and Elvehjem ('43) and Hamilton and Hogan ('44) included pyridoxine in their experimental diets for hamsters, but made no analysis of B<sub>6</sub> deficiency. The experiments embodied in this paper are concerned with the production of vitamin B<sub>6</sub> deficiency in the Syrian hamster.

## EXPERIMENTAL

### *Methods*

With the exception of the preliminary experiment, the work was done on male Syrian hamsters of weanling age (two weeks). They were housed in groups of three to 8

<sup>1</sup>This investigation was supported by grants from the Commonwealth Fund and the National Vitamin Foundation.

animals in metal wire cages with raised screen bottoms. Food and water were offered ad libitum to all but control animals employed for paired feeding. The food, in the form of a moist paste, was placed in small cups. In order to minimize evaporation and splashing, the drinking water was supplied

TABLE 1  
*Composition of diets used*

Component	BASAL MIXTURE			VITAMIN SUPPLEMENTS <sup>1</sup>	
	Diet			Added daily to the diet in mg per 100 gm of basal mixture	
	1	2	3		
Sucrose	75	75	75	Thiamine	0.2
Fibrin	18			Riboflavin	0.3
Casein <sup>2</sup>		18	18	Nicotinic acid	2.5
Corn oil <sup>3</sup>	3	3		Ca pantothenate	2.0
Cottonseed hydrogenated oil <sup>4</sup>			3	Inositol	10.0
Salt mixture <sup>5</sup>	4	4	4	Choline chloride	100.0
				Biotin	0.002
				2-Methyl naphthoquinone	1.0
<i>Given as two drops per week</i>					
<i>Fat-soluble vitamins A, D and E <sup>6</sup></i>					

<sup>1</sup> Provided in the form of halibut liver oil diluted 1:2 with corn oil and fed at a level of two drops per week, with  $\alpha$ -tocopherol included at 0.5 mg per drop.

<sup>2</sup> Vitamin test casein, General Biochemicals, Inc.

<sup>3</sup> Mazola.

<sup>4</sup> Crisco.

<sup>5</sup> No. 2, U. S. P. XII.

<sup>6</sup> The vitamins were synthetic products of the following manufacturers: thiamine, calcium pantothenate, inositol, biotin, riboflavin, nicotinic acid, choline chloride, General Biochemicals, Inc.; 2-methyl-naphthoquinone (Menadione), Ely Lilly Co.; and  $\alpha$ -tocopherol, General Biochemicals, Inc.

from curved graduated glass tubes with a closed top and a turned-up small drinking well at the lower end. The detailed composition of the diets used is presented in table 1. These diets were based on those described by Sarma, Snell and Elvehjem ('46). The hamsters were divided into experimental and control groups. The former groups received no

vitamin B<sub>6</sub>, while the control groups were given daily subcutaneous injections of vitamin B<sub>6</sub>. All the animals were weighed at least once a week. Food and water intake were recorded for each cage 5 to 6 times a week, and for individual animals when kept singly in metabolism cages for the purpose of collecting urine specimens. The animals were observed for a period of 12 to 13 weeks, unless otherwise stated. At the end of the experiment the survivors were killed by inhalation of chloroform. Tissues of autopsied animals were fixed in Bouin's fluid for routine histological sections and stained with Mayer's hematoxylin and eosin. Some tissues were fixed in 20% formalin in preparation for frozen sections. Xanthurenic acid determinations were made on urine specimens collected in metabolism cages according to the method described by Porter, Clark and Silber ('47), using the Coleman Universal Spectrophotometer Model II and a cuvette 13 mm in diameter. The optical density, corrected for the blank value, and multiplied by 3.9 gave xanthurenic acid concentration in milligrams.

### *Preliminary observations*

These were made on 6 hamsters with initial weights ranging from 46 to 68 gm, divided into experimental and control groups of three each and fed diet 1. The animals of the control group were given daily subcutaneous injections of pyridoxine HCl, 50  $\mu$ g per dose. The age of the animals used was not exactly known. It was estimated on the basis of weight to be from three to 4 weeks. There was only moderate retardation of growth, and the appearance of increased amounts of xanthurenic acid in the urine. Autopsy performed after three months of observation showed a striking reduction in the size of the lymphoid tissues, notably the thymus, in the deficient animals. Assuming that the age of the animal may be related to ability to develop the deficiency, the following experiments were carried out on two-week-old weanlings.

*Vitamin B<sub>6</sub> deficiency in hamsters receiving different types of fat in the diet*

Twelve weanling hamsters were divided into 4 groups of three each. Groups 1 and 2 received diet 2, groups 3 and 4, diet 3. The hamsters in groups 3 and 4 received daily subcutaneous injections of 50  $\mu$ g of pyridoxine HCl.

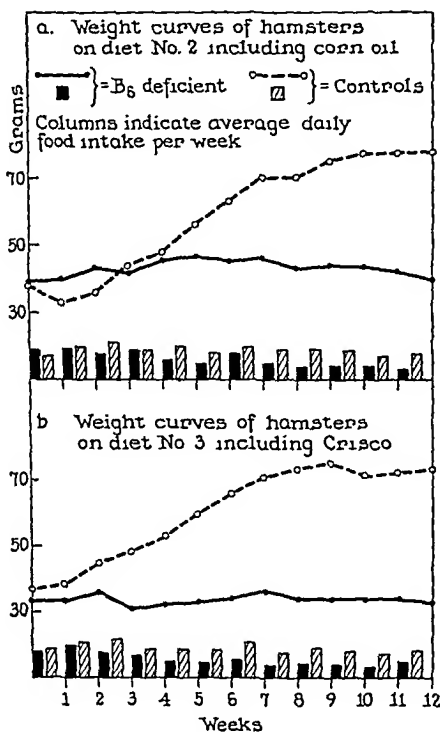


Figure 1

As may be seen from figure 1, most of the animals gained weight during the first two weeks. Later the weight curves began to diverge rapidly. The weights of deficient hamsters on commercial hydrogenated cottonseed oil<sup>2</sup> (diet 3) remained almost stationary, occasionally falling below the initial weight. Those on corn oil (diet 2) showed a slightly

<sup>2</sup> Crisco.

better initial gain. After the 5th week, however, the weights of the corn oil animals declined steadily, although remaining at a persistently higher level than those of the hydrogenated cottonseed oil group. A reduction in food intake took place in the deficient animals after the third week (fig. 1), going hand in hand with the weight curve. When food intake was expressed in relation to body weight it appeared that diminished intake resulted not only from loss of appetite but from diminished body size and possibly from a lowered metabolic rate. The deficient animals also showed reduction in water intake ranging up to 50% and a corresponding decrease in urinary excretion. This was not influenced by the fat composition of the diet. There was no evidence of water retention. Xanthurenic acid excretion by the deficient animals rose from a low of 22 mg in the 4th week to a high of 306 mg per 100 gm of ingested protein in the 8th week. In the controls, the amounts were usually from 10 to 15 mg but in one instance, as high as 30 mg. After a short depletion period the animals began to show very striking deviations from normal development. The symptoms varied somewhat in the individual animals, but there were clear-cut differences between the B<sub>6</sub> deficient hydrogenated cottonseed oil and corn oil groups, particularly with respect to the time of onset of deficiency symptoms.

Two animals of group 1 (hydrogenated oil) rapidly developed other signs of deficiency following arrest of growth, in the second and third week, respectively. The velvety texture of the fur changed to a lustreless gray-brown color, with the ends of the hairs sticking to each other as though they were wet or greasy. There was migrating alopecia, mainly about the head and occasionally on the trunk, the rest of the fur becoming thin and lustreless. The nutritional status rapidly deteriorated in the entire group, with disappearance of fat deposits and progressive muscular weakness. The animals became sluggish and developed a tendency to stay motionless, in a hunched position, sleeping a good deal more than the controls. Some malnourished animals appeared to have a

low body temperature, although this was not measured. They survived for weeks in this condition, not growing and consuming very little food. At the end of 6 weeks one animal exhibited a peculiar dragging gait, one showed spreading of the toes of one foot, and another one had paresis of the right foreleg, persisting until death. Growth retardation remained the only sign of B<sub>6</sub> deficiency in group 2 (corn oil) for 12 weeks. Later a rather striking deterioration set in, characterized by considerable weight loss, poor grooming and lack of lustre of the fur, without alopecia. One hamster had an unsteady, fumbling gait, muscular weakness and spreading of the toes. Two animals succumbed in the 14th week.

Post mortem examination at the end of the experiment confirmed the observations made during life. In addition, reduction in size of the lymphoid tissue was most striking. The degree of involution of the thymus varied somewhat in individual animals. In some animals it could be detected only under the microscope (figs. 6 and 7); the residual gland consisted of tiny lobules of lymphoid tissue embedded in atrophic fat tissue. The cortex disappeared and the lymphocytes were reduced in number, resulting in a relative preponderance of the reticular and vascular structures. Even when the involution of the thymus was less pronounced, diminution in the number of lymphocytes was evident. The lymph-nodes showed a reduction in size and a decrease in the number of lymphocytes and secondary follicles. There were similar changes in the Peyer's patches. The number of lymphocytes in the Malpighian follicles of the spleen was moderately reduced. In the testes there was arrested maturation of spermatozoa, although varying degrees of mitosis were present in the majority of seminiferous tubules. All the control animals showed good growth and development and had at all times a lush brown, soft and abundant fur. Their behavior was marked by liveliness. At three months the weight was about three to 4 times that of the deficient animals (fig. 5). The thymuses were well preserved (fig. 8), with abundant lymphoid tissue in the cortex. They weighed



from 17 to 20 mg. The lymph-nodes were easily identified, the peripheral nodes measuring 1 to 2 mm in diameter. The testes contained mature spermatozoa.

*Paired feeding and treatment of deficient hamsters  
with pyridoxine and corn oil*

Twenty-four hamsters were divided into three groups of 8 each. Their weights varied from 16 to 36 gm. The average weights of groups 1 and 2 (experimental groups) approximately equalled the average weight of group 3 (control group). Diet 3 containing cottonseed oil as the fat was employed. Pyridoxine HCl, in daily doses of 50 µg, was given only to the hamsters of group 3, beginning with the 5th day of the experiment.

Four control animals of group 3 were started on paired feeding as soon as a reduced ad libitum food intake was noted in the B<sub>6</sub> deficient hamsters; namely, by the end of the second week. Their food intake was regulated so as to keep the weight at a level with that of the deficient animals. They were smaller and thinner than normally but not emaciated, and preserved a normal fur. They were active, retained good muscle tone, and merely appeared younger than their mates fed ad libitum. One control hamster, dead after 9 days of paired feeding, showed no atrophy of the lymphoid tissues. Another one, dead after 4 weeks of paired-feeding, revealed moderate atrophy of the lymph-nodes and thymus. More severe lymphoid atrophy was found in a third one dead after 10 weeks of paired feeding. The 4th one, sacrificed at the same time, showed only a slight reduction in the size of the thymus and lymph-nodes. Fatty changes of the liver were found in all the pair-fed controls.

Treatment was begun after 9½ weeks of the deficient regime, and its effect observed for 3½ weeks. Five hamsters of the group including the larger animals were continued untreated and on the usual diet. The remaining animals were treated with the following substances: pyridoxine HCl alone

(group a); pyridoxine HCl and corn oil (group b); and corn oil alone (group c). Pyridoxine HCl was given in daily doses of 50  $\mu$ g, by subcutaneous injection. Corn oil was given orally, replacing the hydrogenated fat in the diet.

The weight curves of the deficient and control animals prior to treatment are shown in figure 2; those obtained during the subsequent period of treatment, in figure 3.

The effect of treatment on weight was most striking in group a. The results with pyridoxine and corn oil were less gratifying, possibly because the animals of group b were smaller than those of group a and appeared to suffer from more advanced nutritional deterioration and fur changes

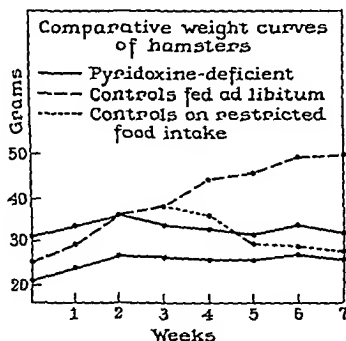


Figure 2

when treatment was begun. Also, they had weighed under 20 gm at weanling age, and developed signs of deficiency more rapidly than the larger weanlings. It is possible that the changes were irreversible and no longer dependent on the absence of pyridoxine. Group c continued to do poorly, showing no response to corn oil. One hamster, which inadvertently had received a single dose of 50  $\mu$ g of pyridoxine HCl, increased in weight from 24 to 30 gm in less than two weeks. This growth-stimulating effect of pyridoxine did not last beyond two weeks. The average daily food intake indicated that in the deficient animals the intake per 100 gm of body weight diminished as the deficiency progressed. Upon institu-

tion of treatment the food consumption increased, most strikingly in group a. It should be noted that food consumption per 100 gm of body weight was higher in the animals treated with pyridoxine after a depletion period than in those which received this vitamin from the start of the experiment. This suggested a metabolism-stimulating effect of pyridoxine during the recovery and active growth period. Under treatment with pyridoxine there was a reduction of 50% or more in the amount of xanthurenic acid in the urine. Corn oil alone had no effect on the excretion of this substance.

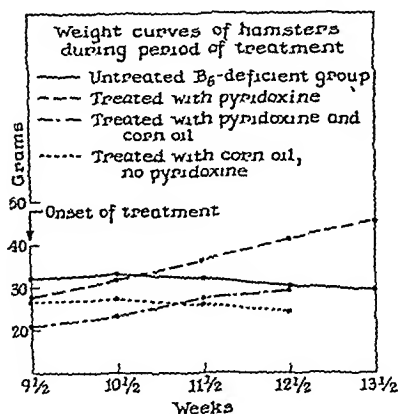


Figure 3

Prior to treatment, growth retardation, fur changes, nutritional deterioration and other clinical signs of deficiency were comparable to those described in the previous experiment. Priapism was noted in two animals about the 6th week, persisting only in one. Some animals showed slight reddening of the skin of the snout or crusting of the upper lip (fig. 10). The animals of group a made a prompt clinical recovery under treatment with pyridoxine. The fur became normal and lustrous within one week, and seemed to increase in amount. The state of nutrition became excellent within a short time. They became more lively and aggressive and their movements recovered quickness and resiliency. They did not

differ from the control animals except for their slightly smaller size (fig. 9). At autopsy there was evidence of regeneration of lymphoid tissue, which was particularly marked in the thymus. The epiphyses of the long bones such as the femur, which showed complete arrest of new bone formation in the deficient hamsters, revealed striking activity, with innumerable newly formed bone trabeculae. In the testes active spermatogenesis was found. In group b, only one hamster showed clinical improvement. The other two persisted in a state of very poor nutrition, although desquamation of the skin on the leg of one cleared up promptly. There was less striking atrophy of the thymus than in the untreated animals, and microscopically there was some evidence of regeneration of lymphocytes. The substitution of corn oil for hydrogenated oil in the diet had no effect on the symptoms of pyridoxine deficiency, the animals of group c resembling the deficient ones in all respects. The animals which received no treatment showed, at the end of 12 to 13 weeks, clinical and post mortem findings of pyridoxine deficiency comparable to those in the previous experiment. It may be mentioned that whenever there was diminished food intake fatty changes were found in the liver, apparently quite independent of the B<sub>6</sub> deficiency.

Comparative anatomical and clinical observations in paired and vitamin B<sub>6</sub> deficient treated and untreated hamsters indicated that the effect of reduced food intake is at least partly responsible for the changes observed in vitamin B<sub>6</sub> deficiency; namely, reduction of fat tissue, atrophy of lymphoid tissue, retardation of skeletal growth and of sexual maturation, and fatty changes in the liver. The alterations of the fur and skin and cachexia, on the other hand, appeared to be independent of the amount of food consumed and directly related to vitamin B<sub>6</sub> deficiency, as these changes responded promptly to treatment with pyridoxine. Moreover, there seemed to be marked alteration of certain metabolic functions of the hamster as manifested by changes in appetite, thirst, excretion of xanthurenic acid, and activity. All these alterations responded to administration of pyridoxine. Corn oil

had no curative action once a vitamin B<sub>6</sub> deficiency had established itself, nor did it promote the therapeutic activity of pyridoxine.

#### DISCUSSION

The only observation recorded in the literature on vitamin B<sub>6</sub> deficiency in the hamster, by Routh and Houchin ('42), seems to have aroused some controversy. The dermatitis described may have been due to biotin deficiency, as pointed out by Cooperman et al. ('43), who were able to prevent similar mouth lesions by the inclusion in the diet of 1  $\mu$ g of biotin per day. On the other hand, Hamilton and Hogan ('44) doubted the need of the hamster for biotin as well as for *p*-aminobenzoic acid. It is possible that the requirements are influenced by the composition of the basal diet and by the relative amounts of other vitamins. Although our purified diet included only 2  $\mu$ g of biotin per 100 gm of diet, no lesions referable to biotin deficiency were observed by us. The mouth lesions occasionally seen were a late manifestation. Prolonged survival of our hamsters also would seem to provide evidence against biotin deficiency, which brings about death in three to 4 weeks (Cooperman et al., '43). The failure to include *p*-aminobenzoic acid and folic acid in our diets did not appear to prevent normal growth and development in the controls; however, the role of these two substances in the nutrition of the hamster requires further study. It should be mentioned also that the amounts of some of the B vitamins used by us were smaller than those employed by Cooperman et al. ('43) and by Hamilton and Hogan ('44). Furthermore, these vitamins were administered in the food, thus allowing a reduced vitamin intake secondary to diminished food consumption. Although the minimum requirements of the hamster for all the vitamins of the B complex are not known, the possibility of multiple deficiencies superimposed on pyridoxine deficiency cannot be completely disregarded.

The syndrome of pyridoxine deficiency in the hamster differs in some respects from that observed in the rat, both

in our experiments with the same diet as used for the hamster and in those published in the literature. While the rate of growth is delayed on a purified diet containing 18% protein, the rat does not show the complete and early arrest of development and the emaciation observed in the hamster under the experimental conditions described. Loss of appetite is one of the earliest and most constant results of pyridoxine deprivation in both species. In our observations, diminution of water intake and urine output was more marked and consistent in the rat than in the hamster. Since the urine of the normal rat contains no xanthurenic acid, in contrast to that of the hamster which usually contains small amounts of this substance, the effect of vitamin B<sub>6</sub> deficiency on xanthurenic acid excretion is more clear-cut in the rat than in the hamster. The most marked divergence in the effect of pyridoxine deficiency between the rat and the hamster is manifest with respect to cutaneous changes. The hamster shows nothing comparable to the classical "rat-acrodynia" first described by György ('34). It may be mentioned in this connection that in our experiments we were unable to produce dermatitis in rats fed a purified diet containing 18% protein and 3% corn oil. When hydrogenated vegetable fat was substituted for corn oil, acrodynia usually appeared about the end of the second month, but did not become as pronounced as has been described by some authors (Sullivan and Nicholls, '40; Antopol and Unna, '42). Severe acrodynia appeared early (after about three weeks) in our experiments with rats on diets containing 30% and 60% protein. Since the need for pyridoxine increases when a high protein diet is given (Miller and Baumann, '45), our observation is not surprising. It remains to be seen whether, by altering the protein content of the basal diet, more accentuated cutaneous alterations can be brought about in the hamster than those observed thus far.

Mouth lesions and priapism occurred less frequently in the hamster than in the rat, while the fur on the trunk and head on a comparable diet remained unaffected in the rat. Epileptiform seizures described in the rat (Chick, El Sadr and

Worden, '40) did not occur in the pyridoxine-deficient hamsters, but there were variable neurologic symptoms such as an ataxic gait or paresis of one extremity and priapism. A relationship between vitamin B<sub>6</sub> and the metabolism of fatty acids has long been recognized, and, although the mechanism is not yet clear, studies on the albino rat indicate that pyridoxine is essential for the maintenance of normal fat metabolism, and that vitamin B<sub>6</sub> and the essential fatty acids complement each other to some extent. Birch ('38) first established the interdependence of vitamin B<sub>6</sub> and the unsaturated fatty acids for their proper utilization by the rat. According to Gross ('40) the dermal manifestations of vitamin B<sub>6</sub> deficiency and fat deficiency (Burr and Burr syndrome) in the rat are identical, and both vitamin B<sub>6</sub> and essential fatty acids are necessary for their cure. The relationship between essential fatty acids and vitamin B<sub>6</sub> was further confirmed by the studies of Salmon ('40, '41), and Schneider, Steenbock and Platz ('40), and, more recently, by Medes and Keller ('47). The latter authors found that alleviation of symptoms of advanced pyridoxine deficiency in rats was attained by either pyridoxine or ethyl linoleate, while for complete cure the administration of both substances was necessary. A similar relationship between pyridoxine and essential fatty acids was suggested by the fact that the symptoms were delayed in hamsters receiving corn oil as compared with those receiving hydrogenated oil as the dietary fat. On the other hand, corn oil was unable to cure in the hamster pyridoxine deficiency once established, or to prevent it completely. This subject needs further investigation. Quantitative and qualitative analyses of body fat similar to those described by Sherman ('47) and Medes and Keller ('47) may throw some light on this problem.

It appears, from the analysis of symptoms produced in pyridoxine-deficient hamsters, and from a comparison between pyridoxine-deficient animals fed ad libitum and paired controls, that not all the symptoms are specific of the deficiency; at least some appear to result from failure to eat

## SUMMARY

Male weanling Syrian hamsters were maintained on a purified diet deficient in vitamin B<sub>6</sub>. This led to arrest of growth after a depletion period of two to three weeks, diminished food and water intake, progressive malnutrition, muscular weakness and changes of the fur. Increased quantities of xanthurenic acid were found in the urine. The deficient animals did not survive beyond 12 to 13 weeks. Autopsy showed loss of fat tissue and marked atrophy of the lymphoid tissues, notably the thymus, even when malnutrition was mild. There was arrest of sexual maturation and bone growth. Pair-fed controls receiving 50 µg pyridoxine HCl daily showed arrest of growth and atrophy of lymphoid tissues, but failed to develop fur changes, muscular weakness, or cachexia. Controls fed ad libitum and receiving daily 50 µg of pyridoxine HCl showed good growth and nutrition and normal activity. Their fur was normal and there was no atrophy of the lymphoid tissues.

Upon treatment of the deficient animals with daily injections of 50 µg of pyridoxine HCl after a depletion period of 9½ weeks, there were in most animals resumption of appetite and growth, deposition of fat and general return to normal behavior and appearance. Fur changes were repaired within one week of treatment. A single injection of pyridoxine HCl seemed to bring about similar results, the effect lasting for about two weeks. Although the presence of unsaturated fatty acids in the diet seemed to delay the onset of deficiency symptoms, the addition of corn oil to the diet of animals in an advanced state of depletion did not have any beneficial effect.

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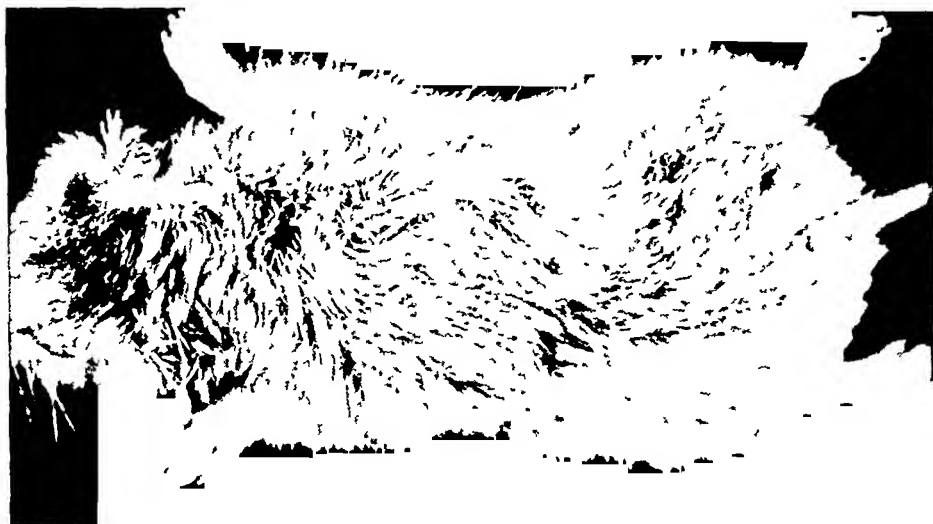
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## PLATE 1

## EXPLANATION OF FIGURES

4 Experiment 2. Hamster, 3½ months old, maintained for three months on vitamin B<sub>6</sub> deficient diet. Note the poor condition of the fur.

5 Experiment 2. A: the same hamster as in figure 4, showing stunted growth. B: Hamster, 3½ months old, which received daily injections of 50 µg of pyridoxine HCl for a period of three months.



4



A



5

## PLATE 2

### EXPLANATION OF FIGURES

6 Experiment 2. Thymus of a vitamin B<sub>6</sub>-deficient hamster, three months old. The section shows tissue from the anterior mediastinum in which extremely atrophic lobules of thymus are embedded (see X). Note also loss of differentiation of cortex and medulla ( $\times 10$ ).

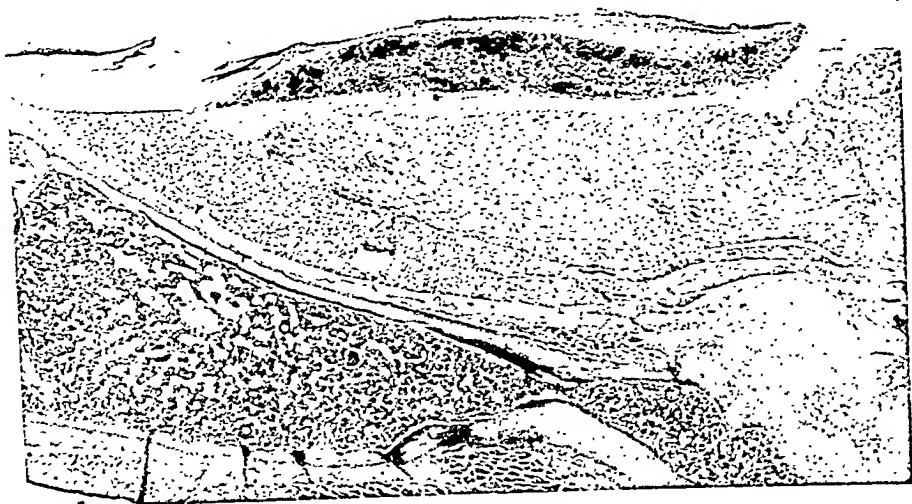
7 Experiment 2. Atrophic thymus of another three-month-old vitamin B<sub>6</sub>-deficient hamster, attached to the posterior surface of the sternum ( $\times 10$ ).

8 Experiment 2. Thymus of a hamster, three months old, which received daily injections of 50  $\mu$ g of pyridoxine HCl for a period of two and one-half months. Note the larger size, and especially the distinct structural differentiation of the thymus ( $\times 10$ ).

B<sub>6</sub> DEFICIENCY IN HAMSTER  
GREGORY SHWARTZMAN AND LOTTE STRAUSS



6



7



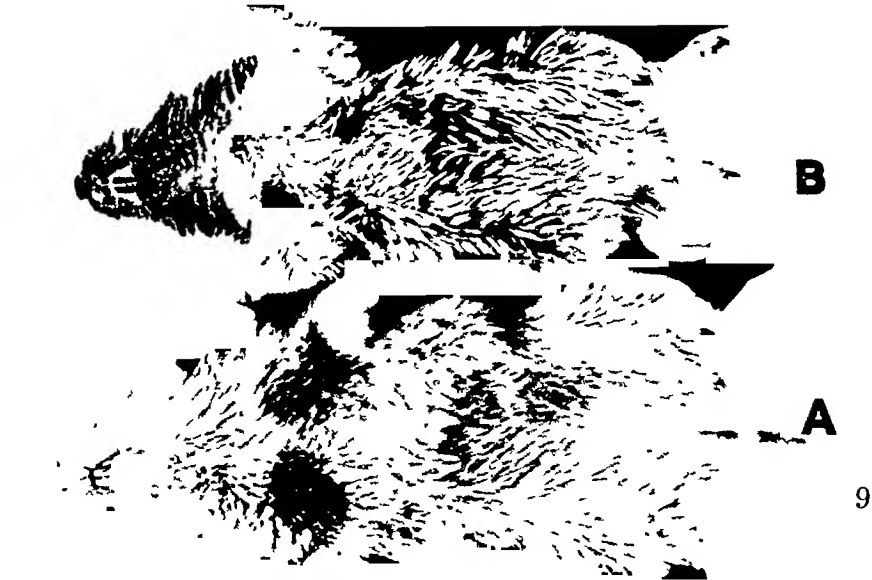
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### PLATE 3

#### EXPLANATION OF FIGURES

9 Experiment 3. Effect of treatment with vitamin B<sub>6</sub> on growth, nutritional status and condition of fur. A: hamster depleted of vitamin B<sub>6</sub> for a period of 9½ weeks, then treated with pyridoxine HCl, 50 µg per day for a period of 3½ weeks. B: hamster of the same age, untreated.

10 Experiment 3. Vitamin B<sub>6</sub>-deficient hamster showing crusted lesion on upper lip. Photograph taken after a depletion period of 12 weeks.



1 2 3 4 5 6 7 8 9 10 11 12 13 14 15







# URINARY EXCRETION OF AMINO ACIDS AND PEPTIDES BY DOGS FED PROTEIN HYDROLYSATES OR AMINO ACIDS

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When proteins or protein derivatives are administered intravenously, they may or may not be utilized by the animal body. Homologous plasma protein (Holman, Mahoney and Whipple, '34) or hydrolysates of protein have biological value after parenteral administration, whereas heterologous plasma protein (Pommerenke, Slavin, Kariher and Whipple, '35) and gelatin (Jacobsen and Smyth, '44; Brunschwig, Nichols and Bigelow, '46) are of less nutritional value under similar conditions. A large fraction of intravenously administered gelatin is excreted in the urine, showing that such materials must be digested before they can be assimilated by the tissues.

When protein hydrolysates are administered by vein a small portion of the amino acids appears in the urine, and the fraction excreted increases as the total dose is increased. Peptides are also lost in the urine (Cox and Miller, '46; Christensen, Lynch and Powers, '46; Frost and Risser, '46). Mixtures containing DL-amino acids are not as well retained as those composed of L-amino acids (Silber, Seeler and Howe, '46).

The present investigation carried out with dogs deals with the urinary loss of amino acids and peptides after intravenous or oral administration of 6 protein hydrolysates and amino

acid mixtures, all which have been employed for the intravenous alimentation of man. Biological values, expressed here as per cent of the total nitrogen retained, have been determined by a procedure devised in collaboration with a U.S.P. subcommittee headed by Dr. J. B. Allison and Dr. D. V. Frost.

#### EXPERIMENTAL

The principles of the nitrogen balance procedure used and the calculations based on them have been described in detail by Allison, Seeley and Ferguson ('47). In such studies, whole egg protein and lactalbumin have a nitrogen balance index of 1.0. In other words, when either of these proteins is fed as the sole source of protein nitrogen to dogs excreting about 100 mg N per kilogram per day in the urine, all of the administered nitrogen is retained in the body. Allison et al. fed whole egg protein during three-day control periods between 4-day tests, whereas in the U.S.P. collaborative study lactalbumin was fed during the control periods. In the present studies nitrogen balance data are expressed in terms of per cent nitrogen retained rather than in nitrogen balance indices, which merely involves a shift in decimal point if the basal urinary nitrogen is assumed to represent endogenous nitrogen.

Adult mongrel dogs, which had been fed a protein-free diet (Silber et al., '46) supplemented with lactalbumin (70 cal. and 120 mg N per kilogram per day) for two to three weeks, were fed or infused intravenously with a protein hydrolysate or amino acid mixture daily for a period of 4 days (120 mg N per kilogram per day). During this period lactalbumin was withheld from the diet. In the oral experiments the solutions were given by stomach tube and in the intravenous experiments a constant rate of infusion (2 mg N per kilogram per minute) was maintained by means of an adjustable rate infusion pump. The dogs were dosed by stomach tube with 350 ml of water per 10 kg body weight at the beginning and again at the end of each 4-day period to facilitate quantitative collection of the urines.

The 6 preparations and the urines, collected under toluene, were assayed for  $\alpha$ -amino nitrogen by the ninhydrin method (Van Slyke, MacFadyen and Hamilton, '43) before and after acid hydrolysis (6 N HCl, 120°C., 6 hr.). When the dogs received lactalbumin the urinary excretion of free  $\alpha$ -amino nitrogen averaged 2.3 mg N per kilogram per day and the

TABLE 1  
*Composition of preparations*

AMINO ACID	PREPARATION					
	A	B	C	D	E	F
	<i>mg/ml</i>					
Arginine	2.2	2.2	1.3	1.5	2.2	3.0
Histidine	0.9	0.9	1.0	1.0	2.4	2.6
Isoleucine	1.4	1.5	2.3	2.2	7.2	6.6
Leucine	4.4	3.1	4.2	4.1	14.9	10.8
Lysine	2.7	2.4	2.5	2.9	10.0	7.5
Methionine	0.5	1.1	1.6	1.1	4.1	2.9
Phenylalanine	2.0	1.7	1.5	2.3	3.9	4.4
Threonine	2.1	2.3	1.6	1.7	1.3	4.2
Tryptophan	0.5	0.3	0.4	0.4	0.8	0.5
Valine	2.4	1.4	2.3	2.4	6.5	8.0
Total N, mg/ml <sup>1</sup>	6.8	7.1	6.5	8.0	13.2	12.8
Free $\alpha$ -amino N, mg/ml <sup>2</sup>	3.1	3.3	2.6	4.0	9.5	10.2
Total $\alpha$ -amino N, mg/ml <sup>2</sup>	5.0	4.7	4.7	5.4	9.2	9.9
Peptides, %	37.6	30.0	44.5	25.0	0.0	0.0

<sup>1</sup> Kjeldahl method.

<sup>2</sup> Free and total  $\alpha$ -amino N by gasometric ninhydrin method before and after acid hydrolysis.

bound  $\alpha$ -amino nitrogen 1.9 mg N per kilogram per day. These values were determined for each dog prior to infusing or dosing, and the values obtained during the test periods corrected by these amounts. Dogs fed protein-free diets have been found to excrete approximately the same amounts of amino acids, and animals fed stock diets excrete little more (Silber et al., '46).

The Kjeldahl total nitrogen, free  $\alpha$ -amino nitrogen, total (free plus peptide)  $\alpha$ -amino nitrogen, and essential amino acid contents of the 6 preparations are listed in table 1. The peptide content is expressed as the per cent of the total  $\alpha$ -amino nitrogen liberated by acid hydrolysis, and the amino acid analyses were carried out by the methods of Stokes, Gunness, Dwyer and Caswell ('45) without additional hydrolysis.

## RESULTS

### *Oral experiments*

When the preparations were administered by mouth, small, almost negligible, fractions of the amino acids were found in the urine, but the excretion of bound amino acids, or peptides, was significantly increased when materials containing peptides were administered. An average of 5.7% of the orally administered peptides was apparently absorbed from the intestinal tract and excreted in the urine. The excretion of amino acids and peptides by two dogs fed each preparation is summarized in table 2. The biological values obtained in nitrogen balance studies, using three or 4 dogs, are also included.

### *Intravenous experiments*

After intravenous infusion at the rate of 2 mg N per kilogram per minute, larger fractions of both amino acids and peptides were excreted (table 2). In general, the loss of peptides in the urine was greater than the loss of free amino acids. This was particularly striking after infusion of preparation C, which gave the lowest value in nitrogen balance experiments.

### *The effect of rate and route of administration on excretion and biological value*

. From table 2 it is clear that the biological value of most preparations was about 25% less when given intravenously

than when given orally. This can be correlated with the urinary loss of approximately 15 or 20% of the infused amino acids and peptides.

It has been suggested (Elman and Weiner, '39) that rapid infusion of protein hydrolysates would be impractical due to the probability of greater urinary loss of amino acids. This

TABLE 2  
*Urinary loss of amino acids and peptides*

PREPARATION	FREE $\alpha$ -AMINO N EXCRETED		BOUND $\alpha$ -AMINO N <sup>1</sup> EXCRETED		TOTAL $\alpha$ -AMINO N <sup>2</sup> EXCRETED		TOTAL N RETAINED <sup>3</sup>	
	%		%		%		%	
<i>After oral administration</i>								
A	1.6	1.3	3.9	5.6	2.4	2.4	61 $\pm$	5.8
B	1.4	1.6	4.3	6.6	2.2	3.1	64 $\pm$	4.8
C	2.6	2.7	2.6	1.5	2.6	2.1	66 $\pm$	2.0
D	4.4	4.2	6.5	5.5	12.6	8.3	67 $\pm$	3.9
E	1.4	0.3			1.0	0.1	73 $\pm$	9.1
F	0.4	0.1			0.8	0.0	61 $\pm$	4.0
<i>After intravenous infusion</i>								
A	12.5	10.7	31.1	29.9	19.5	17.9	45 $\pm$	4.0
B	21.9	18.9	22.6	16.6	24.3	11.3	55 $\pm$	1.0
C	12.9	11.0	46.0	44.6	27.8	25.7	0 $\pm$	13.0
D	8.5	12.3	14.4	14.5	32.3	19.0	40 $\pm$	1.0
E	7.5	3.0			8.8	3.2	57 $\pm$	5.0
F	6.6	7.1			8.1	8.2	43 $\pm$	6.0

<sup>1</sup> Alpha-amino N after hydrolysis minus  $\alpha$ -amino N before hydrolysis.

<sup>2</sup> Alpha-amino N after hydrolysis.

<sup>3</sup> Relative to lactalbumin as 100%; Labeo casein gave values of 70, 72, 73, 76, 76, 76, and 78%.

hypothesis was investigated by infusing preparation E or a mixture of peptide-containing hydrolysates (2A + 1C + 1D) into two dogs at 4 times the rate employed in the previous experiment, and comparing the results with those obtained after administration of these materials to the same dogs both orally and intravenously at the slower rate (table 3). When the daily allotment of these preparations was administered

intravenously in only 15 min. nitrogen retention decreased strikingly, but not as a result of excessive loss of amino acid nitrogen in the urine. The retention of nitrogen decreased from about 60% after oral feeding to about 25% after rapid intravenous feeding, although the excretion of amino acids did not increase more than 15%.

The excretion of peptides after oral feeding was again observed with about 10% passing through the body, presumably unchanged. After intravenous infusion 40% of the peptides could be detected in the urine whether the hydrolysate mixture was given over a one-hour or 15-min. period.

TABLE 3

*Urinary loss of amino acids and peptides after administration of a composite of preparations A, C, and D or preparation E*

PREPARATION ROUTE		PERIOD OF INFUSION	FREE AMINO ACIDS EXCRETED		PEPTIDES EXCRETED		TOTAL N RETAINED <sup>1</sup>	
			%		%		%	
ACD	Oral		0.0	0.0	12.7	8.6	58	62
ACD	I.V. <sup>2</sup>	1 hr.	4.7	5.6	37.9	42.1	40	35
ACD	I.V.	15 min.	14.4	9.4	42.9	40.5	22	32
E	Oral		1.4	0.3			76	56
E	I.V.	1 hr.	7.5	3.0			49	65
E	I.V.	15 min.	12.6	16.9			28	22

<sup>1</sup> Relative to oral lactalbumin as 100%.

<sup>2</sup> Intravenous.

## DISCUSSION

The 6 preparations were approximately equal to casein in biological value when administered orally to dogs, and about 3% of each preparation was excreted in the urine. After intravenous infusion at a relatively slow rate (2 mg N per kilogram per minute) the retention of nitrogen decreased about 25% and the increased loss of nitrogen (Kjeldahl) in the urine could be ascribed largely to the excretion of amino acids and peptides. A 4-fold increase in the rate of infusion caused a decrease of approximately 60% in biological value, or nitrogen retention. The decreased retention of nitrogen,

however, could not be directly related to an increased loss of amino acids, as suggested by Elman and Weiner ('39). Thus, under the present experimental conditions, infusion of the total nitrogen intake in 15 min. was undesirable due to the fact that only 25% of the administered nitrogen could be utilized.

Loss of free amino acids in the urine varied between 5 and 20% when the preparations were infused at a constant rate for one hour. The reason for this variation is not clear, since the retention of total nitrogen could not be correlated with the loss of amino acids in the urine. It appears that the amino acids of certain preparations are more readily excreted unchanged, whereas those of other preparations are more readily deaminated so that more of their nitrogen is lost as urea and ammonia nitrogen. That the "spillage" of amino acids may be related to the biological value of a preparation, as shown for proteins by Pearce, Sauberlich and Baumann ('47), is indicated by an unpublished experiment in which a tryptophan-free amino acid mixture was administered to dogs. When administered orally to two dogs for 4 days, 40% of the nitrogen was retained in the body, but when given by vein all of the nitrogen was lost in the urine. Seventeen per cent of the amino acids was excreted in the urine after infusion of this preparation, although only 5% was lost when tryptophan was included.

The urinary loss of 30% of the peptides infused shows that the peptides of the hydrolysates were not as well retained as the amino acids, which were lost in the urine to the extent of 14%. The fact that increasing the rate of infusion 4-fold did not increase the peptide excretion suggests that the greater loss of peptides in the urine was not solely a result of inability of the kidney tubules to reabsorb peptides from the glomerular filtrate. An alternative explanation is that certain of these peptides were not available to the tissues, due either to their size or composition.

The incomplete utilization of parenterally administered peptides (Christensen et al., '46) is most clearly demonstrated

by the experiments with preparation C. When this hydrolysate was given intravenously to 4 dogs about half of the peptide fraction was found in the urine and the biological value was zero—that is, none of the nitrogen was retained by the body. However, when the same preparation was given orally and thereby exposed to intestinal digestion, it was utilized as well as the other preparations. It appears that certain essential amino acids may have been primarily in the peptide fraction that was lost in the urine after intravenous infusion. This would obviously make the preparation deficient although all essential amino acids were present in the material infused. It is also a possibility that hydrolysate C satisfied the requirements for oral administration to dogs but not those for intravenous administration. We have no reason to assume that the relative amounts of amino acids required in the diet are the same as those required for parenteral feeding. It is not unreasonable to assume that certain amino acids may be utilized less efficiently than others by the intravenous route, and might therefore be required in relatively higher concentrations when the parenteral route is employed.

The data emphasize the necessity of demonstrating that preparations designed for intravenous alimentation can be utilized after parenteral infusion. However, it does not follow that a preparation which is not satisfactorily utilized by laboratory animals, under a given set of conditions, will necessarily be unsatisfactory in man.

Further evidence has been obtained of the unavailability of certain peptides after oral feeding of protein hydrolysates. In these experiments, after feeding three hydrolysates or a mixture of the same hydrolysates, 5 to 10% of the ingested peptides were found in the urine. Since there was little or no increase in peptide amino nitrogen in the urine after the feeding of preparations containing no peptides (E and F) it is reasonable to conclude that the bound  $\alpha$ -amino nitrogen found after feeding peptides was actually peptide in nature, and had its origin in the peptide component of the hydrolysate.



## SUMMARY

1. The urinary excretion of amino acids by dogs was negligible during a 4-day period of oral feeding of protein hydrolysates or amino acid mixtures. After intravenous administration over a period of one hour per day, the loss of free amino acids in the urine ranged from 5 to 20% and there was a decrease in nitrogen retention of approximately 25%.

When the daily allotment was given by vein in only 15 min., the loss of amino acids in the urine increased 10 to 15% and the utilization of nitrogen decreased approximately 60%, due to greater excretion of urea and ammonia nitrogen.

2. After oral feeding of preparations containing peptides, 2 to 11% of the peptide fraction was lost in the urine. After infusion by vein during a one-hour period an average of 30% of the peptides was excreted, in contrast to only 14% of the free amino acids of these preparations. A 4-fold increase in the rate of infusion resulted in no additional loss of peptides in the urine.

3. One preparation, which was utilized approximately as well as casein when administered orally, was found to have no biological value in terms of nitrogen retention when given intravenously. This was apparently related to the excessive urinary loss of its peptides (45%) and its amino acids (12%).

4. The degree of utilization of protein hydrolysates and amino acid mixtures depends upon the method of feeding and upon the rate of infusion.

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# THE EFFECT OF FAT ON CALCIUM AND PHOSPHORUS METABOLISM IN NORMAL GROWING RATS UNDER A NORMAL DIETARY REGIME<sup>1</sup>

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Much work has been done on the effect of fat on calcium and phosphorus metabolism. In most instances either the diet or the animals employed, or both, have been abnormal. The criteria used for assessing results also have varied. Thus Westerlund ('34a, '34b), investigating the influence of tripalmitin, triolein and tributyrin upon calcium metabolism in the adult rat, found that the feeding of tripalmitin caused negative calcium balances while neither triolein nor tributyrin produced such an effect. It is to be noted, however, that the level of calcium fed was kept extremely low to promote the highest degree of calcium utilization. French ('42) and French and Elliott ('43) fed diets containing oleo oil at levels ranging from 0.28% to 45.46% to adult rats. The diets contained the suboptimum level of 0.3% of calcium and a calcium-to-phosphorus ratio of 1:1. They concluded that oleo oil interferes to a slight degree with calcium retention, though the evidence was of a low order of significance.

Knudson and Floody ('40) fed rats a low-fat rachitogenic diet containing a known amount of vitamin D, 1.27% calcium

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and a calcium-to-phosphorus ratio of 5:1. They found that the addition of 5% hardened cottonseed oil promoted better healing in rachitic rats. Larger amounts of fat were not so efficacious. Similarly, Jones ('40) found that the addition to rachitogenic diets of lard to comprise up to 10% increased the bone ash of rachitic rats over a wide range of calcium levels (0.38%–1.0%) and calcium-to-phosphorus ratios (4:1–50:1). Higher levels of lard exerted less influence.

As a result of many studies involving the addition of peanut oil to a high calcium-low phosphorus rachitogenic diet, Booth, Henry and Kon ('42) concluded that fats exert a definite antirachitic effect. They do not exert such an effect in high phosphorus-low calcium diets. Bunkfeldt and Steenbock ('43) fed cottonseed oil as a supplement to various diets and concluded that calcification was depressed in a low-phosphorus rachitogenic ration (phosphorus 0.075%). When phosphorus was optimum (0.25%), calcification was increased. Further increases in phosphorus content indicated a much smaller beneficial effect of fat, which was quite independent of the calcium-to-phosphorus ratio.

While fat most certainly affects the utilization of calcium and phosphorus, it appears that the absolute level of calcium and of phosphorus, their ratio to each other, the fat used and the condition of the animal are important factors. It seemed to us desirable to determine the influence of fat on calcium and phosphorus utilization when a normal diet is fed to normal, growing rats.

#### EXPERIMENTAL

The percentage composition of the ration used was egg albumen (cooked), 18.0; dextrinized starch, 73.4; and Jones and Foster ('45) salt mixture, 3.6. To this was added 5% of the fat to be used—completely hydrogenated cottonseed oil (HCSO), peanut oil (PNO) or coconut oil (CNO). One group was given an additional 5.2% of dextrinized starch in lieu of fat, the salt mixture being dropped to 3.4% so as to preserve

a constant ratio of calories to minerals. Daily vitamin supplements of 80  $\mu$ g thiamine hydrochloride, 160  $\mu$ g riboflavin, 120  $\mu$ g pyridoxine hydrochloride and 100  $\mu$ g of calcium pantothenate were given. Vitamins A and D were supplied by one drop of cod liver oil twice each week. The rations provided approximately 0.6% of calcium and 0.4% of phosphorus.<sup>3</sup>

Twelve 60-gm male rats were placed on each diet. In the case of the hydrogenated cottonseed oil, however, the feces and urine collections were from 10 rats, as the urine from two of the animals was inadvertently discarded after the completion of the feeding. The rats were kept in individual mesh-bottomed metabolism cages, each of which was set in a funnel in which had been placed a copper screen for the separation of feces and urine. Collections were started following a two-day preliminary feeding period. Feces were removed from the screen each day and the cage bottom, screen, and funnel washed with hot water acidified with sulfuric acid to a pH of 4.6 to 4.8. Twice each week, the urine, plus washings, was collected from beakers placed under the funnels and stored in glass jars at 7 to 8°C. until analyzed.

Food wastes and contamination with excreta were held to negligible quantities by the use of a specially prepared feeding device. A Franke-type cup was placed in the center of a can having a diameter of about 5 inches, the can being cut off at an angle of approximately 30° and the whole assembly covered with quarter-inch mesh and suspended on the side of the cage. By this arrangement, sleeping on the feeding cup was prevented and scattered food fell through the mesh into the large can, permitting subsequent recovery and weighing.

At the conclusion of a 28-day feeding period, the rats were killed with chloroform and the femurs removed for bone ash determinations. Calcium and phosphorus determinations were made of the diets and samples of the feces and urine.

<sup>3</sup>The actual percentage of calcium and phosphorus was as follows: HCSO diet — Ca 0.68, P 0.42; CNO diet — Ca 0.63, P 0.40; PNO diet — Ca 0.65, P 0.40; no-fat diet — Ca 0.61, P 0.44.

The methods of Morris, Nelson and Palmer ('31) were used in these determinations.

## RESULTS

### *Calcium and phosphorus balances*

Data on food intakes, weight gains and calcium and phosphorus determinations are given in table 1. Initially an attempt was made to control rigidly and equalize the amount of food eaten. This did not prove feasible, possibly because of a difference in appetite for the various fats. Sufficient

TABLE 1  
*Results of chemical determinations and feeding trials*

CATEGORY OF INTEREST	D I E T			
	HCSO	CNO	PNO	No-fat
Food intake (gm)	297.3	307.7	295.5	311.0
Weight gain (gm)	121.4	115.5	116.5	117.7
<i>Calcium</i>				
In food (gm)	$2.0 \pm 0.08^1$	$1.9 \pm 0.04$	$1.9 \pm 0.06$	$1.9 \pm 0.12$
In feces (gm)	$0.8 \pm 0.07$	$0.7 \pm 0.05$	$0.6 \pm 0.08$	$0.4 \pm 0.23$
In urine (gm)	$0.1 \pm 0.07$	$0.1 \pm 0.02$	$0.2 \pm 0.05$	$0.2 \pm 0.12$
<i>Phosphorus</i>				
In food (gm)	$1.3 \pm 0.05$	$1.2 \pm 0.02$	$1.2 \pm 0.08$	$1.4 \pm 0.10$
In feces (gm)	$0.2 \pm 0.02$	$0.3 \pm 0.03$	$0.3 \pm 0.04$	$0.3 \pm 0.08$
In urine (gm)	$0.2 \pm 0.06$	$0.1 \pm 0.04$	$0.1 \pm 0.04$	$0.1 \pm 0.08$
Femur ash (%)	$61.9 \pm 1.90$	$62.8 \pm 0.70$	$62.0 \pm 1.80$	$62.0 \pm 1.00$

<sup>1</sup> Standard deviation.

control was maintained, however, so that the mean variation of the amounts eaten was not large. All animals gained well.

Calculated values of the proportion of calcium and phosphorus in the feces and urine and that which was retained are given in table 2. The significance of the differences between the means of calcium and phosphorus in the feces and in the urine for each fat and the comparable mean for the no-fat diet were tested, using Fisher's ('30) modification of "Student's" *t* test.

Fecal calcium increased with increase in the melting point of the fat used. The animals on the no-fat diet excreted the least calcium in the feces. The differences between the means of the HCSO and CNO diets, respectively, and that of the no-fat diet were highly significant; between the mean of the PNO diet and the no-fat diet, the differences were below the significant level.

In general, urinary calcium varied inversely to fecal calcium, though the variations were of smaller magnitude. Hence, retentions of calcium varied nearly as greatly as the fecal

TABLE 2  
*Partition of dietary calcium and phosphorus*

CATEGORY OF INTEREST	D I E T			
	HCSO	CNO	PNO	No-fat
<i>Calcium</i>				
Fecal (%)	42 $\pm$ 2.5 <sup>1</sup>	35 $\pm$ 3.2 <sup>1</sup>	30 $\pm$ 4.5	27 $\pm$ 4.4
Urinary (%)	3 $\pm$ 0.1 <sup>1</sup>	7 $\pm$ 1.9	7 $\pm$ 3.1	8 $\pm$ 1.8
Retention (%)	55 $\pm$ 2.6 <sup>1</sup>	59 $\pm$ 2.7 <sup>1</sup>	63 $\pm$ 1.7	65 $\pm$ 4.3
<i>Phosphorus</i>				
Fecal (%)	18 $\pm$ 2.9	27 $\pm$ 3.4 <sup>1</sup>	26 $\pm$ 4.8 <sup>1</sup>	20 $\pm$ 1.8
Urinary (%)	19 $\pm$ 2.1 <sup>1</sup>	6 $\pm$ 1.7	5 $\pm$ 1.8	6 $\pm$ 2.1
Retention (%)	63 $\pm$ 4.4 <sup>1</sup>	67 $\pm$ 4.1 <sup>1</sup>	69 $\pm$ 4.9 <sup>2</sup>	74 $\pm$ 3.6

<sup>1</sup> The difference between this value and that of the fat-free diet is highly significant ("t" value exceeds that tabulated for  $P = 0.01$ ).

<sup>2</sup> The difference between this value and that of the fat-free diet is significant ("t" value exceeds that tabulated for  $P = 0.05$ ).

calcium, but in inverse order. Again the difference between the means was highly significant in the case of the HCSO and CNO diets, but not significant in the case of the PNO diet.

The excretion of fecal phosphorus revealed no constant relationship either to the characteristics of the fat used or to the presence or absence of fat in the diet. Animals on the CNO and PNO diets excreted more phosphorus than those on the no-fat diet. There was little difference between results on the HCSO and no-fat diets.

The very high excretion of phosphorus in the urine of rats on the HCSO diet was the only noteworthy feature of urinary phosphorus excretion. It lowered the retention of phosphorus to a value less than that obtained on any other diet; consequently retentions of phosphorus varied in the same manner as retentions of calcium. The difference between the means of the HCSO and CNO diets and that of the no-fat diet were highly significant; that between the means of the PNO diet and the no-fat diet was significant.

### *Excretion of calcium soaps*

Holt, Courtney and Fales ('20) suggested that certain instances of poor calcium retention were due to the formation of insoluble calcium soaps. In order to discover the extent

TABLE 3  
*Neutral fat and fatty acids of feces*

CATEGORY OF INTEREST	D I E T			
	HCSO	CNO	PNO	No-fat
Dry weight of feces (gm)	25.36	18.22	16.19	17.94
Neutral fat in feces (gm)	2.28	0.58	0.82	0.60
Free fatty acids in feces (gm)	10.64	1.13	1.96	1.44
Total (as triglycerides) (gm)	13.41	1.76	2.86	2.10

to which this reaction might be responsible for the higher fecal calcium noted in rats fed the fat-containing diets, we attempted to determine the quantity of calcium soaps excreted in the feces. Several methods were tried and that of Augur, Rollman and Denel ('47) was finally used, although it did not prove entirely satisfactory. The results are given in table 3.

Rats on the no-fat diet excreted considerable quantities of neutral fat and soaps. These, as Augur et al. have pointed out, must be regarded as of "metabolic" origin.

The animals on the CNO diet excreted even less neutral fat and soaps; hence it would appear that all of the fat in



their diet was absorbed, since the quantities found in the feces were of such small magnitude that they could all be accounted for as metabolic. This agrees with the findings of Hoagland and Snider ('43a) that coconut oil is 98.9% digestible at the 5% level. The relatively high fecal excretion of calcium noted in rats on this diet cannot be attributed to the formation of calcium soaps.

Rats on the PNO diet excreted slightly larger quantities of neutral fat and soaps than did rats on the no-fat diet.

Particularly noteworthy is the much greater amount of feces passed by animals on the HCSO diet than that by animals on the other diets. All of this increase was accounted for by increases in the amounts of free fat and soap excreted, which was expected in view of the higher fecal excretion of calcium observed.

### *Bone formation*

Data on the analysis of the femurs for ash is given in table 1. It can readily be seen that, at least in the 28 days of feeding, the differences in mineral metabolism obtained on the diets did not effect the formation of the bones.

### DISCUSSION

The inclusion of 5% fat in the diet increased fecal calcium. From the work of Holt, Courtney and Fales ('20) one might conclude that the rather large increase in fecal calcium in rats on the HCSO diet was probably due to poor absorption of calcium stearate formed in the digestive tract. Hoagland and Snider ('43b) have also reported on the poor absorption of tristearin and stearic acid. The HCSO was completely hydrogenated, hence it would have contained a large percentage of stearate. The large amounts of free fatty acids (soaps) noted in the feces of these animals would tend to substantiate such a theory.

On the other hand, the excretion of free fatty acids (soaps) by rats on the CNO and PNO diets was much smaller, so that it seems improbable that such a reaction was a factor in the

increases of fecal calcium noted in these two groups of animals. Calcium stearate is utilized by the rat, to a limited extent at least, as Boyd, Crum and Lyman ('32) have shown. It would seem likely that the much smaller amounts of calcium stearate which might have been formed on the CNO and PNO diets were largely absorbed.

Bergeim ('26) demonstrated that calcium is excreted into the intestine. Hence, the presence of calcium in the feces may be due to lack of absorption or to excretion. The extent of excretion varies over a considerable range, depending on the diet. Following a period of feeding calcium citrate, Steggerda and Mitchell ('46) found fecal calcium to be as much as twice the calcium intake. Greenberg ('45) found that about 18.0% of an injected dose of radioactive calcium appeared in the feces, whereas 32.5% appeared when the same amount was administered orally. It was not possible in the present experiments to determine whether the increased calcium in the feces of those rats fed the CNO and PNO diets was due to decreased absorption, to increased excretion, or to both.

It does seem that the increased excretion of fecal calcium imposed no strain on the economy of calcium in the body except, possibly, in those rats on the HCSO diet. Urinary calcium remained nearly constant except for the latter group. Bone ash apparently was not affected in any group, which is interesting in view of the marked variation in retention from group to group.

Phosphorus, too, is excreted into the intestine, as Bergeim ('26) has shown, and its presence in the feces may be due to lack of absorption or to excretion. Though the addition of CNO and PNO to the diet increased fecal phosphorus, HCSO did not have such an effect. This may have been due to the removal of interfering calcium from the intestine, as calcium stearate, when HCSO was fed. With the CNO and PNO diets there was little, if any, increase in soap formation but there was an increase in calcium excretion, possibly as a phosphate, thus accounting for the observed increase in

phosphorus excretion. Certainly there was no indication that the inclusion of fat, per se, increased fecal phosphorus.

Urinary phosphorus, like urinary calcium, was not much affected except in the group on the HCSO diet. Though fecal phosphorus was the lowest in this group, urinary phosphorus was over three times as great as in the other groups. Such an effect might have been due to a higher plasma concentration after absorption, giving rise to an excessive excretion before the phosphorus could be used, or to a lack of calcium ions which would aid in fixing phosphorus in the tissues.

The intimate relationship existing between calcium and phosphorus metabolism was evident in the values obtained for phosphorus retention. In spite of the irregular and divergent effect of the different fats on phosphorus excretion in the feces and urine, its retention varied in a manner similar to that of calcium.

When calcium is in excess, as in many rachitogenic diets, and the fat is partly composed of triglycerides of long-chain, saturated fatty acids, the formation of calcium soaps, with consequent lowering of phosphorus lost in the feces, may bring about some improvement in phosphorus utilization. This may possibly explain the results of Knudson and Floody ('40) and of Jones ('40). The observation of Booth, Henry and Kon ('42) that the equicaloric inclusion of peanut oil in the diet lowered both the fecal excretion of phosphorus and the urinary excretion of calcium is in direct contrast to our results. It was possibly due to the higher calcium and lower phosphorus content of their rachitogenic diet (calcium 0.9%, phosphorus 0.18%) and the formation of calcium soaps when fat was included.

#### CONCLUSIONS

1. The inclusion in a normal diet of 5% fat (completely hydrogenated cottonseed oil, coconut oil, or peanut oil) increased the fecal excretion of calcium.
2. When fats containing many of the poorly absorbed glycerides of the long-chain, saturated fatty acids were fed,

the increase in fecal excretion was probably due to the formation of relatively insoluble calcium soaps. When this was the case, the excretion of phosphorus in the feces was not increased.

3. When fats containing mostly the more rapidly absorbed glycerides of long-chain, unsaturated or short-chain, saturated fatty acids were fed, the increase in fecal excretion of calcium was of lesser magnitude. It did not seem to be due to the formation of calcium soaps and it apparently carried phosphorus with it, since fecal phosphorus was also increased.

4. The excretion of calcium and phosphorus in the urine was affected only indirectly by the presence of fat in the diet. When the fecal excretion of calcium was considerably increased there was a decrease in the urinary excretion of calcium but a marked increase in the urinary excretion of phosphorus.

5. The cumulative result of the inclusion of 5% fat in the diet was a decrease in the retentions of calcium and of phosphorus which roughly paralleled the increase in the fecal excretion of calcium.

6. The inclusion of 5% fat in the normal diet, well supplied with calcium and phosphorus and having an optimum calcium-to-phosphorus ratio, did not apparently affect the formation of bone in healthy, growing rats.

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	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100	

## PURIFIED AMINO ACIDS AS A SOURCE OF NITROGEN FOR THE GROWING RAT<sup>1</sup>

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More than 10 years ago Rose and co-workers demonstrated that 10 amino acids are required by the rat for rapid growth, while 12 other amino acids are dispensable. An extensive review of this subject appeared in 1938 (Rose, '38). At about the same time Rose ('37) presented tentative minimum requirements for each of the 10 indispensable amino acids for rapid growth of the rat. A reinvestigation by Rose and Womack ('46) when an improved ration was used showed that the phenylalanine requirement proposed earlier (0.7%) was somewhat low, and that 0.9% was required for maximum growth. This requirement was reduced to nearly half when the ration contained about 0.6% L-tyrosine (Womack and Rose, '46b).

Subsequent work by this group (Borman et al., '46) confirmed their earlier observation (Scully and Rose, '30) that the young rat can synthesize arginine from materials ordinarily available in the ration, but not at a rate commensurate with the requirements for rapid growth. Therefore, arginine was classified as an essential dietary component for optimum

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growth. Using an improved ration in which the composition of the amino acid mixture, vitamin supplement, and gross constituents were altered, Womack and Rose ('47) investigated the nutritional role of glutamic acid, hydroxyproline and proline. They found that hydroxyproline and proline were dispensable, but that the latter could partially replace the arginine required for rapid growth. Arginine, glutamic acid and proline were mutually interconvertible in the rat but at different rates, as shown by the decreasing order of their influence upon growth. However, the best rates of growth were obtained when all three amino acids were included. Later studies (Rose et al., '48) indicated that glutamic acid was stimulatory when added to a ration containing only the 10 essential amino acids, but its omission from a ration containing 19 amino acids did not reduce the growth rate significantly.

Evidence of the existence in proteins of an unidentified growth stimulant for rats was presented by Womack and Rose ('46a), who observed that the growth rate of animals receiving their best amino acid ration was far less than that of rats receiving a ration containing 18% casein supplemented with 0.2% methionine (3.5 gm per day compared to 5.1 gm per day). Partial replacement of the amino acids by proteins such as casein improved the growth rate while the corresponding acid hydrolysates did not. It was concluded therefore that young rats require an unknown growth factor contained in intact proteins. Whether this is identical with "strep-ogenin," the peptide which has been reported to be a growth stimulant for certain bacteria and mice (Woolley, '45), was not established.

The present communication deals with studies begun nearly three years ago in an effort to formulate a mixture of purified amino acids which would support growth rates in weanling rats approximating those obtainable with equivalent amounts of a protein such as casein. For this purpose the minimum requirements for the essential amino acids as proposed by



Rose in 1937 and modified subsequently (Womack and Kade, '44) served as a starting point.

#### EXPERIMENTAL

Weanling male rats of the Sprague-Dawley strain, 21 days old and within a 5 gm weight range, were used. They were placed in individual cages and fed ad libitum for 28 days. In most of the groups the daily food consumption was recorded to permit the calculation of the protein efficiency of the different rations. The animals were weighed at weekly intervals.

Each group consisted of two to 4 animals, with three in a majority of the groups. Whenever a suggestive observation was made the experiment was repeated with a larger number of animals, using the same ration, and the results of all such trials are presented together. The growth rates and protein efficiency values given in the tables represent the averages together with the range or the standard error of the mean. When possible, the "t" test was applied and a P value of 0.05 or less was considered statistically significant.

The rations used had the following basic percentage composition: Corn oil 5, salts IV (Phillips and Hart, '35) 4, monosodium phosphate monohydrate<sup>4</sup> 0.63, vitamin mixture 2, amino acids, casein<sup>5</sup> or acid hydrolysate of casein<sup>6</sup> as indicated, and sucrose to make up to 100%. The 2 gm of the vitamin mixture in a sucrose base contained: Thiamine hydrochloride 0.2 mg, riboflavin 0.3 mg, pyridoxine hydrochloride 0.25 mg, niacin 1.5 mg, inositol 10 mg, choline chloride 100 mg, calcium *d*-pantothenate 2 mg, biotin 10 µg, and pteroyl-glutamic acid 20 µg. Fat-soluble vitamins were given once each week to each rat in the form of two drops of a modified halibut liver oil to provide a daily intake of 400 I.U. of vitamin

<sup>4</sup> To supply the phosphorus contained in 18% casein for which the salt mixture was designed.

<sup>5</sup> Vitamin-test casein, General Biochemicals, Inc., was used throughout these experiments.

<sup>6</sup> See footnote 5.

A, 4 I.U. of vitamin D, 0.7 mg of  $\alpha$ -tocopherol, and 0.06 mg of 2-methyl-1, 4-naphthoquinone.

The composition of the essential amino acid mixture "A" used in the preliminary studies is given in table 1. To provide 5.6% of the physiologically active essential amino acids in

TABLE 1  
Composition of the essential amino acid mixtures "A" and "B"<sup>1</sup>

AMINO ACID	PHYSIOLOGICALLY ACTIVE FORM	AS USED <sup>2</sup>	NITROGEN
	%	%	%
L-Lysine	1.0		
monohydrochloride		1.24	0.190
L-Histidine	0.4		
monohydrochloride mono-hydrate		0.54	0.108
L-Arginine	0.2		
monohydrochloride		0.25	0.066
DL-Tryptophan	0.2	0.20	0.027
DL-Phenylalanine	0.7 (0.9)	0.70 (0.9)	0.059 (0.076)
L-Leucine	0.8	0.80	0.086
DL-Isoleucine	0.5	1.0	0.107
DL-Threonine	0.5	1.0	0.118
DL-Methionine	0.6	0.6	0.056
DL-Valine	0.7	1.4	0.168
Total	5.6 (5.8)	7.73 (7.93)	0.985 (1.002)

<sup>1</sup> The figures in parentheses refer to essential amino acid mixture "B."

<sup>2</sup> Amino acids as supplied by the manufacturers (mostly Merck and Interchemical) were used after checking their purity by microbiological methods (Henderson and Snell, '48). Various samples of commercially available DL-isoleucine showed from 35 to 100% of the expected activity for *Lactobacillus delbrückii* 3, presumably because of contamination with DL-alloisoleucine (Smith and Greene, '48). The samples used in these studies ranged from 76 to 80% pure by this criterion and correspondingly larger amounts were included in the mixtures. It was assumed that the nitrogen of this impurity was available to the rats as non-essential amino acid nitrogen.

the proportions proposed by Rose and co-workers (Rose, '37; Womack and Kade, '44), it was necessary to add 7.73% of the amino acids used, in order to correct for the inactive D-forms of isoleucine, valine and threonine, and the HCl and water of crystallization of the salts of the basic amino acids.

## RESULTS

The results of preliminary studies with amino acid-containing rations are presented in table 2. When essential amino acids at a level of 5.6% were the sole source of nitrogen, the growth rate was very poor (0.41 gm per day). The addition of 2% glutamic acid improved the growth rate to about 1.3 gm per day; a further increase in glutamic acid to 6% resulted in little improvement. On the other hand, the addition of 2%

TABLE 2

*Results of preliminary studies with amino acid rations<sup>1</sup>*

NITROGENOUS COMPONENTS	N CONTENT OF RATION	NO. OF ANIMALS	GROWTH RATE	
			Average	Range
	%			gm/day
5.6% EAA	0.98	3	0.41	0.28-0.57
5.6% EAA + 2% GA	1.17	3	1.3	1.2-1.4
5.6% EAA + 6% GA	1.55	6	1.6	1.4-1.8
5.6% EAA + 2% GA + 2% casein }	1.48	3	2.7	2.6-2.8
5.6% EAA + 2% GA + 2% AHC }	1.48	3	2.3	2.2-2.3
5.6% EAA + 0.2% DL-phenyl- alanine + 5.8% GA }	1.55	11	2.5	2.1-2.9

<sup>1</sup> EAA = Essential amino acid mixture "A" shown in table 1.

GA = L-Glutamic acid.

AHC = Acid hydrolyzed casein.

glutamic acid and 2% casein or acid hydrolyzed casein gave growth rates of 2.7 and 2.3 gm per day, respectively. To determine whether this beneficial effect was due to the supply of an essential amino acid in which the mixture was limiting, a number of experiments with smaller numbers of animals were conducted wherein the essential amino acids were added individually and in groups of two and three. The results were negative in all cases (data not presented) except that the addition of 0.2% DL-phenylalanine resulted in an improved rate of growth (2.5 gm per day). At about the same time

Rose and Womack ('46) reported that 0.9% phenylalanine was required with their revised ration. Accordingly the essential amino acid mixture "A" was modified to contain 0.9% phenylalanine and the resulting mixture "B" (table 1) was used in all subsequent experiments reported.

A number of modifications involving the other major ingredients of the ration were made in an effort to improve the growth rates further. Isocaloric replacement of sucrose by 25 gm of corn oil, doubling the vitamin content, the replacement of corn oil and a part of the sucrose by dextrin, lard and agar as used earlier by Rose and Womack ('46), the addition of 5% cellufLOUR or replacement of the sucrose by dextrin, resulted in no improvement. The observation of Borman et al. ('46) on the adverse effect of high levels of fat was confirmed. In several instances during the course of this work 2% lyophilized liver was used and a slight stimulation in growth was observed, probably due to the protein supplied. Antipernicious anemia liver preparations administered intraperitoneally did not stimulate the growth of rats receiving a number of the amino acid rations.

The effect of varying the levels of glutamic acid and the essential amino acid mixture was next studied (table 3). When the glutamic acid level was increased from 5.8% to 15.8% (rations 1 and 3), there was a slight increase in the average rate of growth ( $2.5 \pm 0.07$  and  $2.7 \pm 0.08$  gm per day), but this difference was not statistically significant ( $t = 1.83$ ,  $P = 0.087$ ). It is to be noted, however, that comparatively high levels of glutamic acid seem to be well tolerated by the growing rat. Therefore, this amino acid was used in many subsequent experiments for adjusting the nitrogen content of the rations to the desired level. The results with rations 1 and 2 (table 3) suggested a slight depression in growth rate when the essential amino acid mixture was increased from 5.8% to 7.7% at the expense of glutamic acid. Doubling the level of the mixture (compare rations 3 and 4) resulted in a depression of the growth rate from  $2.7 \pm 0.08$  to  $2.3 \pm 0.11$

gm per day, the difference being statistically significant ( $t=2.96$ ,  $P=0.014$ ).

Since the rate of growth of rats receiving rations containing amino acids was much less than that of rats receiving equivalent amounts of casein, a number of experiments (see table 4) were conducted to determine whether this poor performance was due to the lack of streptogenin. For this purpose, an acid hydrolysate of casein was prepared as previously described (Henderson et al., '47). The hydrolysate was tested microbiologically (Woolley, '48) and found to be

TABLE 3

*Effect of varying levels of the essential amino acid mixture "B" and glutamic acid*

RATION NO.	NITROGENOUS COMPONENTS	N CONTENT	NO OF ANIMALS	GROWTH RATE	PROTEIN EFFICIENCY <sup>1</sup>
		%		gm/day	
1	5.8% EAA <sup>2</sup> + 5.8% GA	1.55	11	2.5 ± 0.07	3.23
2	7.7% EAA + 2.3% GA	1.55	2	2.3	3.00
3	5.8% EAA + 15.8% GA	2.5	9	2.7 ± 0.08	2.08
4	11.6% EAA + 5.25% GA	2.5	4	2.3 ± 0.11	2.02

<sup>1</sup> Protein efficiency represents the gain in body weight in grams per gram of protein ( $N \times 6.25$ ) consumed.

<sup>2</sup> See footnote, table 2.

free of streptogenin; however, it contained the expected amounts of all essential amino acids other than tryptophan (Henderson and Snell, '48). When a ration containing 20% of this hydrolysate supplemented with 0.2% DL-tryptophan and 0.2% L-cystine was fed to rats, the growth rate averaged  $4.25 \pm 0.13$  gm per day, while the rats fed an equivalent level of casein (19%) grew at the rate of  $4.61 \pm 0.15$  gm per day. The difference between these growth rates was not significant ( $t=1.82$ ,  $P=0.082$ ). Replacement of part of the hydrolysate by 5% intact casein or 5% egg albumin purified according to the method of Woolley ('46) gave average growth rates of  $4.55 \pm 0.14$  and  $4.40 \pm 0.09$  gm per day, respectively. From

these results it was concluded that the lack of streptogenin does not limit the growth of young rats significantly.

In view of the fact that the growth rate and protein efficiency vary with the level of protein in the ration, it seemed desirable to maintain a constant level of nitrogen in all rations. In all subsequent experiments, therefore, a nitrogen level of 2.5% was used. This amount is the same as that provided by 19% air dried casein, which is generally considered adequate for optimum growth in the rat. The results of these experiments are presented in table 5.

TABLE 4  
*Comparison of acid hydrolyzed casein and intact proteins*

NITROGENOUS COMPONENTS	NO OF ANIMALS	GROWTH RATE <i>gm/day</i>	PROTEIN EFFICIENCY <sup>1</sup>
19% Casein	11	4.61 $\pm$ 0.15	2.67 $\pm$ 0.03
20% AHC + 0.2% DL-tryptophan + 0.2% L cystine	11	4.25 $\pm$ 0.13	2.59 $\pm$ 0.06 <sup>2</sup>
14.5% AHC + 0.2% DL tryptophan + 0.2% L-cystine + 5% casein	11	4.55 $\pm$ 0.14	
14.5% AHC + 0.2% DL-tryptophan + 0.2% L cystine + 5% egg albumin	3	4.40 $\pm$ 0.09	

<sup>1</sup> See footnote 1, table 3.

<sup>2</sup> Based on 8 of the 11 animals.

While the rate of growth with rations containing 5.8% essential amino acids plus 15.8% glutamic acid was  $2.7 \pm 0.08$  gm per day, the isonitrogenous substitution of 5% casein for glutamic acid increased the growth rate significantly to  $3.4 \pm 0.13$  gm per day. Since earlier experiments had failed to show any improvement with higher levels of the essential amino acids, singly or collectively, it seemed probable that this beneficial effect of casein was due to its non-essential amino acids. This impression was confirmed when a similar rate of growth ( $3.4 \pm 0.12$  gm per day) was obtained with the

addition of 6.12% of an arbitrary mixture of non-essential amino acids.<sup>7</sup>

Many experiments of an exploratory character, with two animals in each group, were conducted to ascertain which of these non-essential amino acids was responsible for the observed growth stimulation. By adding the amino acids singly and in various combinations, at the expense of glutamic acid, it was found that glycine, cystine, alanine, serine, proline, and asparagine (or aspartic acid) had negligible effects on the growth rate; figures for these groups are therefore not reported in table 5.

In preliminary experiments a stimulatory effect of L-tyrosine was noted when it was added to rations containing only the essential amino acids and glutamic acid. In the presence of 0.75% arginine hydrochloride, tyrosine increased the growth rate from 2.6 to 3.6 gm per day (rations 4 and 5, table 5). The further addition of 0.5% DL-serine and 1.2% DL-alanine (rations 6 and 7) did not produce any significant change. An attempt was made (rations 13 and 14) to determine whether the growth stimulatory effect of tyrosine could be duplicated by additional amounts of phenylalanine. The basal ration used resembled ration 9 but contained no tyrosine. When 0.5% of either L-tyrosine or DL-phenylalanine was added, the growth rate was approximately the same. Further work, with larger numbers of animals and wider ranges of tyrosine and phenylalanine concentrations, is needed before it can be determined whether the stimulatory action of tyrosine is due to an inadequate phenylalanine intake or to a limited rate of formation of tyrosine from phenylalanine.

In the presence of 14.3% glutamic acid (rations 1 and 4) additional arginine had no effect on growth. Comparison of rations 9 and 12 indicated that when all but 2% of the glutamic

<sup>7</sup> Five-tenths per cent DL-serine, 0.7% glycine, 1.4% L-tyrosine, 0.2% L-cystine, 0.9% L-proline, 1.22% L-asparagine and 1.2% DL-alanine. This same mixture was used at various levels in the other experiments described here.

TABLE 5  
*Effect of non-essential amino acids on growth<sup>1</sup>*

RATION NO.	NITROGENOUS COMPONENTS <sup>2</sup>	NO. OF ANIMALS	GROWTH RATE gm/day	PROTEIN EFFICIENCY <sup>3</sup>
1.	5.8% EAA + 15.8% GA	9	2.7 ± 0.08	2.08 ± 0.05
2.	5.8% EAA + 5% casein + 8.8% GA	4	3.4 ± 0.13	2.32 ± 0.05
3.	5.8% EAA + 6.2% GA + 6.1% NEAA	4	3.4 ± 0.12	2.29 ± 0.01
4.	5.8% EAA + 14.3% GA + 0.5% L-arg. HCl	3	2.6 (2.4-3.0) <sup>4</sup>	2.1 (1.9-2.3)
5.	5.8% EAA + 13.1% GA + 0.5% L-arg. HCl + 1.4% L-tyrosine	3	3.6 (3.6-3.8)	2.44 (2.29-2.63)
6.	5.8% EAA + 12.4% GA + 0.5% L-arg. HCl + 1.4% L-tyrosine + 0.5% DL-serine	3	3.8 (3.6-4.0)	2.51 (2.41-2.56)
7.	5.8% EAA + 10.4% GA + 0.5% L-arg. HCl + 1.4% L-tyrosine + 0.5% DL-serine + 1.2% DL-alanine	3	3.8 (3.4-4.0)	2.37 (2.37-2.37)
8.	5.8% EAA + 9.2% NEAA + 0.5% L-arg. HCl	5	3.5 ± 0.18	2.48 ± 0.04
9.	5.8% EAA + 7.85% NEAA + 0.5% L-arg. HCl + 2% GA	9	4.1 ± 0.12	2.50 ± 0.05
10.	5.8% EAA + 6.56% NEAA + 0.5% L-arg. HCl + 4% GA	3	3.4 (3.1-3.8)	2.38 (2.26-2.48)
11.	5.8% EAA + 4.9% NEAA + 0.5% L-arg. HCl + 8% GA	3	3.8 ± 0.20	2.39 (2.06-2.64)
12.	5.8% EAA + 8.85% NEAA + 2% GA	3	3.0 (2.9-3.1)	2.37 (2.31-2.48)
13.	5.8% EAA + 6.74% NEAA (no tyrosine) + 0.5% L-arg. HCl + 2% GA + 0.5% L-tyrosine	3	3.8 (3.4-4.0)	2.43 (2.36-2.52)
14.	Same as ration 13, but 0.5% DL-phenylalanine instead of tyrosine	3	3.5 (2.9-4.2)	2.21 (2.0-2.6)

<sup>1</sup> Nitrogen content of all the rations was 2.5%.

<sup>2</sup> EAA = essential amino acid mixture "B" shown in table 1. NEAA = non-essential amino acid mixture (see text). See also footnote, table 2.

<sup>3</sup> See footnote 1, table 3.

<sup>4</sup> Range.



acid was replaced by the non-essential amino acid mixture additional arginine was beneficial.

In another series of experiments (rations 8 to 11) the effect of varying the concentration of glutamic acid was studied, the level of the non-essential amino acid mixture being altered to adjust the nitrogen content to 2.5% in each case. Comparison of rations 8 and 9 suggested a stimulation of growth by 2% glutamic acid. Higher levels (rations 9-11) did not produce quite as good growth, possibly because of a simultaneous reduction in the other non-essential amino acids. This question was examined more critically by Rose, Oesterling and Womack ('48). They found that the removal of glutamic acid from a diet containing 19 amino acids resulted in a slight inhibition of growth which was of doubtful statistical significance.

It is to be noted that ration 9 gave the best growth of all the amino acid-containing rations studied. The average growth rate was  $4.1 \pm 0.12$  gm per day and the protein efficiency averaged  $2.5 \pm 0.05$  gm per gram of protein consumed. The corresponding figures for a 19% casein ration were  $4.61 \pm 0.15$  gm per day and  $2.67 \pm 0.03$  gm per gram of protein. This improvement on ration 9 seemed to be the result of the growth-stimulating effects observed earlier with 0.5% arginine hydrochloride, 2% L-glutamic acid and 1.8% L-tyrosine (supplied by the non-essential amino acid mixture).

In all of these experiments it was observed that the rats receiving the free amino acids grew at reduced rates during the first week compared with the rats receiving casein. This was presumably due to the poor palatability of the amino acids as evidenced by the lower food consumption. As they became accustomed to the rations, however, the animals ate well and the growth rate during the third and 4th weeks was improved. However, the rats appeared incapable of completely overcoming the effects of the initial handicap. To obviate this difficulty forced feeding was used, and thereby the food intake of the amino acid-fed rats was equalized with that of the casein-fed rats. The ration was homogenized in a

TABLE 6  
*Effect of forced, paired-feeding of amino acid and casein rations<sup>1</sup>*

EXP. NO.	GROUP NO.	NITROGENOUS COMPONENTS	METHOD OF FEEDING	NO. OF ANIMALS	GROWTH RATE <i>gm/day</i>	PROTEIN EFFICIENCY <sup>2</sup>
I	1	19% casein	Ad libitum	3	4.8 (4.8-5.6) <sup>3</sup>	2.78 (2.67-2.92)
	2	19% casein	Forced, paired with group 1	4	4.8 (4.0-5.4)	2.77 (2.34-3.13)
	3	5.8% EAA + 4.9% NEAA + 8% GA + 0.5% L-arg. HCl	Ad libitum	3	3.6 (3.3-3.8)	2.39 (2.06-2.64)
	4	5.8% EAA + 4.9% NEAA + 8% GA + 0.5% L-arg. HCl	Forced, paired with group 1	4	4.4 (4.3-4.6)	2.46 (2.39-2.51)
II	5	19% casein	Ad libitum	3	4.25 (3.9-4.4)	2.63 (2.16-2.72)
	6	5.8% EAA + 7.85% NEAA + 2% GA + 0.5% L-arg. HCl	Ad libitum	3	4.1 (3.7-4.5)	2.53 (2.42-2.69)
	7	5.8% EAA + 7.85% NEAA + 2% GA + 0.5% L-arg. HCl	Forced, paired with group 5	3	4.3 (4.2-4.5)	2.57 (2.48-2.63)

<sup>1</sup> EAA = essential amino acid mixture "B." See table 1, as well as footnote, table 2. NEAA = non-essential amino acid mixture. See text.

<sup>2</sup> See footnote 1, table 3.

<sup>3</sup> Range.

small volume of water using a Potter-Elvehjem homogenizer to give a thick slurry. It was administered by stomach tube in three feedings at 5-hour intervals daily. The results of two such paired feeding experiments (see table 6) showed that the increased intake of the amino acid rations resulted in a better growth rate, approaching that of rats receiving casein rations *ad libitum*. Forced feeding *per se* did not have any adverse effect, as can be seen from the data relating to groups 1 and 2. Comparison of groups 3 and 4 and groups 6 and 7 showed that the forced feeding of the amino acids did not reduce the protein efficiency values. It should be noted that these values closely paralleled the growth rates at a given nitrogen level. For example, the correlation coefficient,  $r$ , for protein efficiency values and growth rates in table 5 is 0.67. From these observations made on more than 200 rats it appears that food consumption figures are of no special value in studies of this type.

#### DISCUSSION

The results obtained in the course of this investigation confirm the observations made by Rose and associates regarding the inadequacy of 0.7% phenylalanine (Rose and Womack, '46) and the improved growth resulting from the addition of the non-essential amino acids to adequate amounts of the essential amino acids (Rose et al., '48). It is not possible to make a quantitative comparison of the present results and those obtained by the above workers. The chief points of difference are with regard to arginine and tyrosine. Borman et al., ('46) stated in a footnote that 0.25% arginine hydrochloride was adequate for growth and that larger percentages were not more effective under the conditions of their tests. In the present series of experiments, additional arginine was found beneficial. Similarly, Womack and Rose ('46b) observed that the addition of tyrosine failed to accelerate the growth rate when the diet contained an adequate amount (0.9%) of phenylalanine. The studies reported here indicate a growth stimulation with tyrosine.

A number of differences in the experimental rations and the way in which the experiments were designed may account for these apparent discrepancies. In the experiments described here the essential amino acids were used at the levels tentatively suggested as minimum (Rose, '37; Womack and Kade, '44), contributing only 5.8% of physiologically available forms. Rose and Womack (Rose and Womack, '46; Womack and Rose, '47) have used the essential amino acids at levels of 25 to 100% above these minimum requirements, giving approximately 8.6% of the active forms. As a result of this and the rather low levels of non-essential amino acids fed, the ration of non-essential amino acid nitrogen to essential amino acid nitrogen in the mixtures used by these workers was somewhat lower than those in the rations we used. Finally, in all except their recent experiments Rose and co-workers (Borman et al., '46; Womack and Rose, '47; Rose et al., '48) employed rations containing 31% fat.

The non-essentiality of tyrosine was established by experiments in which limited growth was obtained with no dietary source of this amino acid. Abundant evidence has been presented of its formation from phenylalanine in the rat. Whether the conversion of phenylalanine to tyrosine is rapid enough to permit optimum rates of growth is not known. This is a possible explanation for the stimulatory action of tyrosine in these experiments. Womack and Rose ('46b) established the phenylalanine requirement and the extent to which tyrosine will substitute for phenylalanine in experiments where the growth rate did not exceed 2 gm per day in most cases. It seems possible that (a) the phenylalanine requirement may be more than 0.9% for optimum growth without dietary tyrosine or (b) the rate of formation of tyrosine may become limiting as the growth rate increases. Further, the capacity for this conversion may be affected by the nature of the ration. Such a concept is in keeping with the recent findings of Rose and co-workers ('48) regarding the need for glutamic acid. The results reported here could be explained by either of the above alternatives, but it is not

possible to distinguish between them from the data thus far obtained. It should be noted that in no case did the growth exceed 3.0 gm per day without tyrosine or additional phenylalanine, while growth rates of 3.6 gm per day were obtained with 12 amino acids. Similar considerations may explain the failure of Borman et al., ('46) to obtain better growth with 0.6% arginine than with 0.2%. As has been pointed out (Womack and Rose, '47), the stimulatory effect of arginine was very small until improvements were made in the rations. It seems possible that still other of the so-called non-essential amino acids may be shown to be stimulatory as the growth rates approach more closely those observed with good quality protein.

Although the improvements made in the amino acid mixtures resulted in growth rates as high as 4.1 and 4.4 gm per day under conditions of ad libitum and forced feeding, respectively, it should be noted that the growth performances were still slightly inferior to those of rats receiving casein. Computations from the data on the 11 animals receiving the casein diet (table 4) and the 9 rats fed the best amino acid ration (group 9 in table 5) show that the difference between these groups is significant, the P value being 0.03. Further work is needed to find an amino acid mixture that will support a growth rate as good or better than casein. The inferiority of the present amino acid rations may possibly be due to the presence of the unnatural D-isomers. In the formulation of these rations it has been assumed that the D-amino acids serve to supply non-essential amino acid nitrogen. At the levels used they may not produce any symptoms of toxicity but may cause a slight depression of growth. On a ration containing a mixture of synthetic (racemic) amino acids, Wretling ('48) obtained a growth rate of 0.33 gm/day at a 10% level and 0.89 gm/day at 20%, while at 30 and 40% levels the animals lost weight. He concluded that synthetic amino acids contain some toxic material. In these laboratories an alkaline hydrolysate of casein was prepared by autoclaving with barium hydroxide. Microbiological assays (Henderson

and Snell, '48) for the essential amino acids in the hydrolysate showed that arginine and threonine were completely destroyed and several others, particularly valine, histidine and isoleucine, suffered partial destruction. This hydrolysate was fed to rats at a 20% level, supplemented with the essential amino acids to correct for these losses. The animals ate very little of the ration and died within a week. This was probably not due to D-amino acids alone but to the presence of toxic products formed during the hydrolysis. There is no report of any rat growth studies employing only the natural forms of the amino acids. However, Brand and Bosshardt ('48) have reported that mice fed a mixture of L-amino acids duplicating the composition of  $\beta$ -lactoglobulin grew at the same rate as those receiving a corresponding amount of the native protein. When certain DL-amino acids were used in the mixture, the growth response was impaired.

In these laboratories, Maddy and Elvehjem ('49) have fed a balanced mixture of 16 amino acids to mice and obtained a rate of growth closely approaching that obtained with casein. Their work also emphasizes the importance for optimum growth of adding some of the non-essential amino acids. Possibly an imbalance of the amino acids in the mixtures employed in these studies could account for their inferiority as a source of nitrogen for the growing rat. Grau ('48) has presented evidence that the lysine requirement for the growth of the chick is a direct function of the protein content of the ration. Work in this laboratory (Hankes et al., '48) on the niacin-tryptophan relationship has shown that slight increases in the concentrations of certain amino acids cause marked reduction in the growth rate of rats, again suggesting the importance of a proper balance of amino acids in rations where the levels of certain dietary essentials are marginal.

#### SUMMARY

A study was made of the growth of weanling rats receiving rations containing mixtures of purified amino acids, acid

hydrolyzed casein and intact casein. Daily food consumption was recorded and data on the protein efficiency of the different rations are presented.

The growth rate of young rats fed 20% acid hydrolyzed casein supplemented with 0.2% DL-tryptophan and 0.2% L-cystine was not significantly less than that of rats receiving an equivalent amount of intact casein, offering no support for the presumption that the rat requires a source of streptogenin under these conditions.

Improved rations containing 18 amino acids to provide a nitrogen level of 2.5% gave average growth rates of 4.1 and 4.4 gm per day under conditions of ad libitum and forced, paired feeding, respectively. These values represent 80 to 90% of the growth rate of rats receiving ad libitum a ration containing casein at an isonitrogenous level. Evidence presented indicates that the improved growth rate was the result of increasing the level of arginine hydrochloride to 0.75% and adding 2% L-glutamic acid and 1.8% L-tyrosine to a ration containing other "non-essential" amino acids and the minimum levels of the 10 essential amino acids.

The probable reasons for the slight inferiority of the amino acid-containing rations to the casein-containing ration are discussed.

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# THE CHOLINE REQUIREMENT OF THE BABY PIG

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THIRTY FIGURES

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In a previous paper it was established that the baby pig on a 30% casein ration requires dietary choline, and the deficiency syndrome was described (Johnson and James, '48a). The object of the experiments reported in this paper was to determine the dietary choline level essential for optimum growth and for the physiological well-being of the pig.

To determine the choline requirement it is necessary to consider the methionine (or other methyl donor) content of the diet. A choline deficiency was produced in the baby pig using a diet containing approximately 0.8% methionine, based on microbiological assays of casein (Henderson and Snell, '48; Horn et al., '46; Stokes et al., '45) to 1.0% based on chemical assays (Block and Bolling, '45). This suggests that in the baby pig the methylation of aminoethanol by the methyl groups from methionine does not take place to any considerable extent (compare with the chick as discussed by Jukes, '41).

## EXPERIMENTAL

Two-day-old Duroc-Jersey pigs from the University farm were used in this study. The technique of feeding and care of the animals has been described (Johnson, James and Krider, '48b). The composition of the basal ration is given

in table 1. As in previous experiments, 2% sulfasuxidine<sup>1</sup> was included in the ration to inhibit intestinal synthesis. Two experiments were conducted; one using ad libitum feeding to establish the range of the requirement and the second using

TABLE 1  
*Composition of the basal diet*

	%
Casein (Labco, vitamin-free)	30.0
Glucose (cerelose)	37.4
Mineral salts <sup>1</sup>	6.0
Lard	26.6

These materials were made up into a "milk" containing 4% lard (liquid basis) and the following amounts of vitamins<sup>2</sup> per liter:

Vitamin A	2000 I.U.	Pyridoxine	2.67 mg
Vitamin D <sub>2</sub>	200 I.U.	Ca-pantothenate	8.00 mg
Alpha-tocopherol acetate	1.0 mg	Inositol	26.00 mg
2-methyl-1,4-naphthoquinone	0.26 mg	PABA	2.67 mg
Thiamine	0.65 mg	PGA	0.052 mg
Riboflavin	1.33 mg	Biotin	0.01 mg
Nicotinic acid	2.67 mg		

Choline chloride was added as indicated in the text. Reticulogen<sup>3</sup> was added at the rate of 0.25 ml/day in test 2.

<sup>1</sup> See Johnson et al., '48b.

<sup>2</sup> Thiamine hydrochloride, riboflavin, pyridoxine hydrochloride, calcium pantothenate, biotin, nicotinic acid and  $\alpha$ -tocopherol acetate were supplied by Hoffmann-La Roche, Inc., Nutley, New Jersey, through the courtesy of Dr. J. C. Bauernfeind. Pteroylglutamic acid was supplied by the Lederle Laboratories Division, American Cyanamid Co., Pearl River, New York, through the courtesy of Dr. T. H. Jukes. Inositol was supplied by the A. E. Staley Manufacturing Co., Decatur, Illinois. Hyflavin (a highly water-soluble form of riboflavin) was supplied by Endo Products, Inc., Richmond Hill, New York.

<sup>3</sup> Reticulogen was supplied by Eli Lilly and Co., Indianapolis, Ind.

paired feeding to eliminate any effects due to differences in food intake within the limits of this range.

In the first experiment 4 groups of three pigs each were fed ad libitum the basal ration plus choline chloride at the

<sup>1</sup> Sulfasuxidine was supplied by Sharp and Dohme, Inc., Glenolden, Pa., through the courtesy of Dr. S. F. Scheidy.

following levels based on the dry matter of the ration: Group I—0.0%, II—0.05%, III—0.10% and IV—0.20%. The experiment was continued for 8 weeks. At its conclusion representative pigs from each group were sacrificed and microscopic sections were made of their livers, kidneys, nerves, and skeletal muscles.

From the results of experiment 1 the dietary requirement for choline chloride appeared to be between 0.05% and 0.1%. Therefore, in the second experiment these two levels of choline were compared by pair feeding 6 pairs of pigs for 8 weeks, as in experiment 1. Reticulogen, a liver extract material, was added to the basal ration in this experiment at the rate of 0.25 ml daily to supply an essential growth factor or factors, as it had been found by the present authors in previous work that the basal diet was deficient in this respect (Neumann et al., '48). All 12 of the pigs in experiment 2 were sacrificed and microscopic sections made of their tissues as in experiment 1. In addition, ether extract determinations were made of the livers.

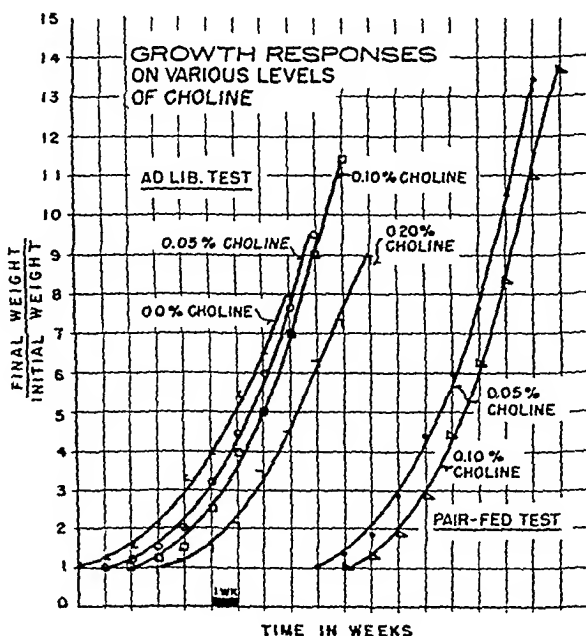
## RESULTS

The average growth curves of the groups of pigs from both experiments are plotted in chart 1. The rates of gain of the 4 groups in experiment 1 are not significantly different, although the difference between groups I and III is indicative of a growth effect ( $P=0.08$ ). However, this effect as shown in experiment 2 and as reported previously (Johnson and James, '48a) is probably due to an increased food intake. Similar findings have been reported by Foá, Weinstein, and Kleppel ('48) for the rat. These workers found no effect of a choline deficiency on the growth of the rat when food consumption was equalized. From the data in table 2 it is apparent that the 0.05% and 0.10% choline groups made increasingly more rapid gains on less dry matter consumed than the negative control group, although the "P" value between the gains of the 0.0% and 0.1% choline groups is only 0.08%.

Pictures of two representative pigs from each of the first three groups in experiment 1 are given in plate 1. Pigs in figures 1 and 2 are from the "0.0% choline" group; those in 3 and 4 from the "0.05% choline" group; and the animals in 5 and 6 from the "0.1% choline" group. The pigs in figures 1 to 4, and in particular 2 and 4, show the gross

CHART 1

Graph showing the average growth responses on the various levels of choline. Note similarity of the growth pattern, regardless of level fed or method of feeding used, i.e., freedom from a plateau in the growth curve even on the choline-free diet.



symptoms of choline deficiency. They are unthrifty and have poor conformation, appearing short-legged and pot-bellied. These pigs lacked coordination in their movements (as also reported by Ellis, Madsen and Miller, '43) and seemed to lack proper rigidity in the joints, particularly in the shoulders (note also pigs 8 and 9, plate 1, Johnson and James, '48a).

The microscopic sections of nerve and skeletal muscle failed to show any differences between groups.

The performance records of the baby pigs in experiment 2 are given in table 3. The two groups showed no difference in rate or economy of gain.

Three pairs of pigs from experiment 2 are pictured in plate 2. Pigs in figures 7, 9, and 11 received 0.05% choline, while those in figures 8, 10 and 12 received 0.1%. The pigs on the higher level of choline were thriftier in appearance and had better haircoats than their pair mates on the lower level.

The histological findings with respect to the livers and kidneys of pigs from both experiments are illustrated in

TABLE 2

*Response of baby pigs fed various levels of choline ad libitum for 56 days*

DIETS FED	BASAL + 0.00% CHOLINE	BASAL + 0.05% CHOLINE	BASAL + 0.10% CHOLINE	BASAL + 0.20% CHOLINE
No. of pigs fed	3	3	3	3
Ave. initial weight (kg)	1.67	1.60	1.55	1.70
Ave. final weight (kg)	13.49	15.27	17.56	15.36
Ave. $\frac{\text{final weight}}{\text{initial weight}}$ (kg)	8.07	9.53	11.69	9.22
Dry matter consumed (kg)/kg gain	1.22	1.11	1.07	1.18

plates 3, 4 and 5. In plate 3, figures 13, 14 and 15 are photomicrographs of sections of the livers of pigs in experiment 1 receiving 0.0, 0.5 and 0.10% choline, respectively. Figures 16 and 17 are liver sections from pair-fed pigs in experiment 2 on 0.05 and 0.10% choline, respectively. Note that on the choline-free diet (fig. 13) the hepatic cells of the liver have become greatly distended with fat and the nuclei are pushed over to one side. The livers of the animals on the 0.05% level of choline contain slightly more fat globules than those from the pigs on the 0.10% level. In addition, there was a slight but significant difference in ether extract content of the livers.

Renal damage appeared more serious than the liver condition. The photomicrographs (plates 4 and 5) illustrate the degree of damage to the glomeruli and the tubular epithelium. On the 0.0% level all glomeruli shown are occluded and apparently non-functional (fig. 18), as contrasted with the glomeruli seen in figure 20 from a pig on the 0.1% choline level and in figure 21 from a pig on an adequate farm ration.

The photomicrographs were made of the cortical region of the kidney in all cases, as damage in this area was more pronounced. The kidneys of the pigs on the 0.05% choline level show less damage than those on the 0.0% level but are

TABLE 3

*Effect of 0.05 and 0.10% choline in baby pig diets (equal feed intake for 56 days)*

DIETS FED	BASAL DIET + 0.05% CHOLINE	BASAL DIET + 0.10% CHOLINE
No. of pigs fed	6	6
Ave. initial weight (kg)	1.60	1.59
Ave. final weight (kg)	21.32	21.79
Ave. $\frac{\text{final wt.}}{\text{initial wt.}}$ (kg)	13.32	13.70
Ave. liver weight (dry) (gm)	146.72	137.17
Ave. ether extract in liver (%)	5.80	5.20
Ave. liver ether extract/kg body wt.	1.34	1.16
Dry matter consumed (kg)/kg gain	1.167	1.140

definitely abnormal. This is true in both the ad libitum feeding experiment (fig. 19) and in the paired feeding experiment (fig. 22), while the kidneys of the pigs on the 0.10% level are normal (figs. 20 and 23). The photomicrographs in plate 5 illustrate at higher magnification the kidneys on both the ad libitum and the paired feeding experiments when less than 0.10% choline was included in the diet.

#### DISCUSSION

An analysis of the data presented in this paper shows that the very young pig requires choline even in the presence of a substantial amount of methionine. In this respect the inter-

relationship of choline and methionine in the baby pig is similar to that in the chick rather than that in the rat. Jukes ('40) showed that methionine will not prevent the symptom of perosis seen in choline deficiency in the chick; that is, it will not methylate aminoethanol (Jukes, '41) to form choline as it does in the rat (Du Vigneaud, Chandler, Cohn and Brown, '40). Recently McKittrick ('47) has reported that for optimum growth of 4-week-old chicks 0.5% methionine is required when optimum choline (0.55%) is included in the diet, and that 0.10% choline is required when optimum methionine (0.75%) is included in the diet. The baby pigs in our experiments received approximately the same amount of methionine (0.8%) as that which allowed optimum growth with 0.1% choline in these chick experiments. Similarly the pigs showed a definite choline requirement of the same magnitude (0.1%) as that which McKittrick reported for the chick.

However, Treadwell ('48) has recently reported that in the young (50 gm) rat the requirement for methionine on a choline-free diet is 1.3 to 1.8%. While there is no choline requirement at this level of methionine, when the diet contains only 0.8% methionine the young rat has a definite need for choline. With optimum choline the methionine requirement of the rat is 0.5-0.6% (Womack and Rose, '41).

From these data it would seem necessary to undertake further experiments involving still higher methionine levels to determine whether the baby pig can synthesize all of its required choline in the presence of adequate methionine or whether there is an absolute dietary requirement for choline.

#### SUMMARY

1. On a "synthetic milk" ration containing approximately 0.8% methionine (30% casein) the baby pig requires 0.1% choline in its diet.

2. It appears that in the young pig, as in the chick, the ability of methionine to supply methyl groups for the synthesis of choline from aminoethanol may be limited. The

methionine requirement of the baby pig must be established before this can be determined.

3. When the food intake is equalized choline does not have a growth stimulating effect.

4. Choline deficiency in these baby pigs resulted in gross symptoms of unthriftiness, poor conformation (short-legged and pot-bellied), lack of coordination in movements and a characteristic lack of proper rigidity in the joints, particularly the shoulders. The pigs also showed typical fatty infiltration of the livers and characteristic renal glomerular occlusion and some tubular epithelial necrosis.

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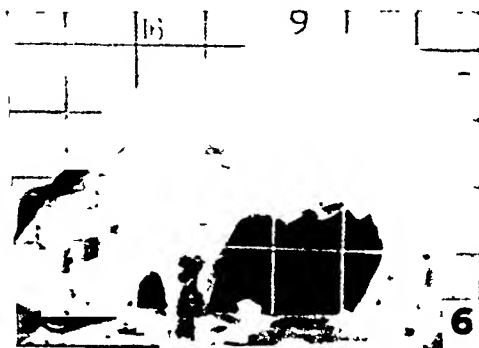
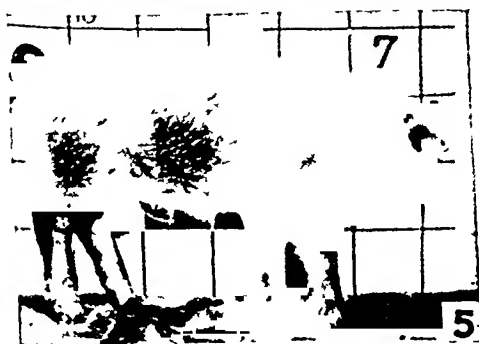
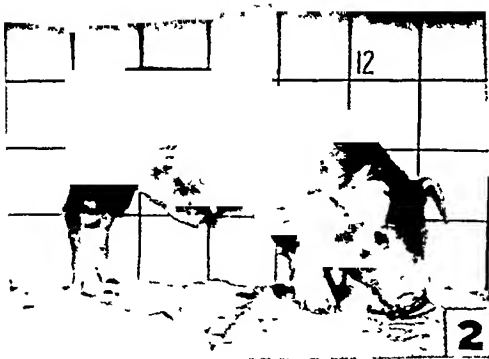
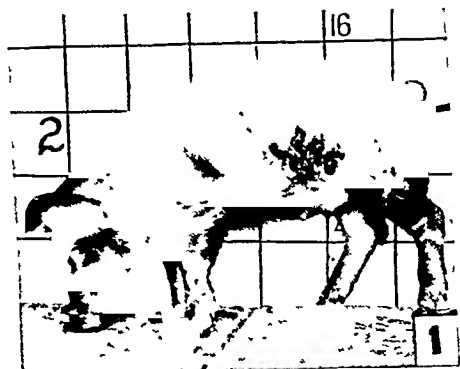


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## PLATE 1

### EXPLANATION OF FIGURES

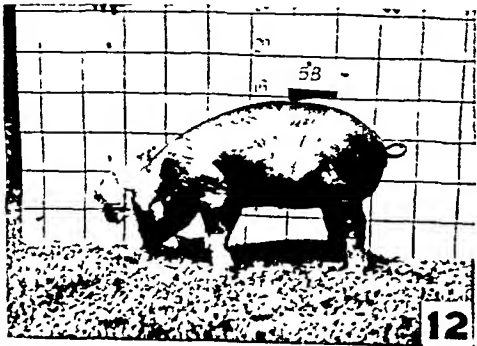
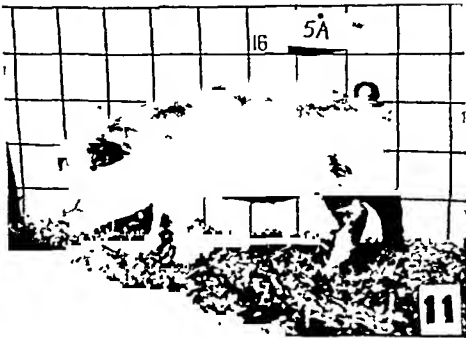
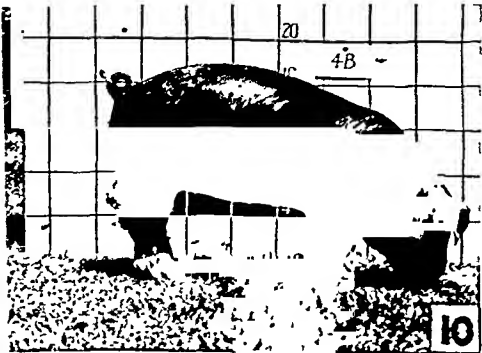
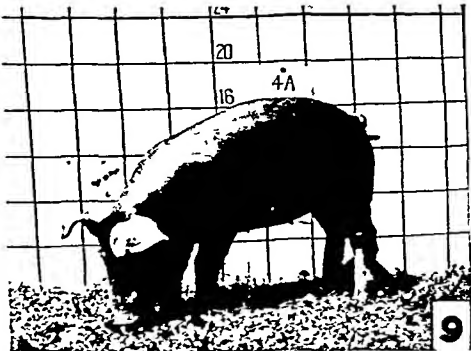
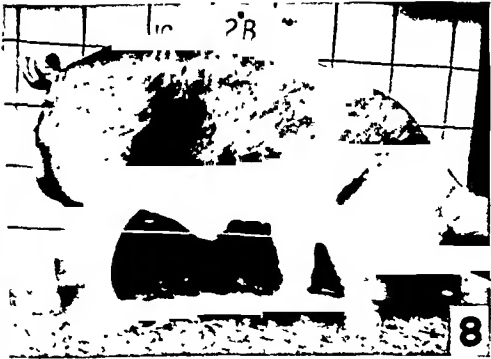
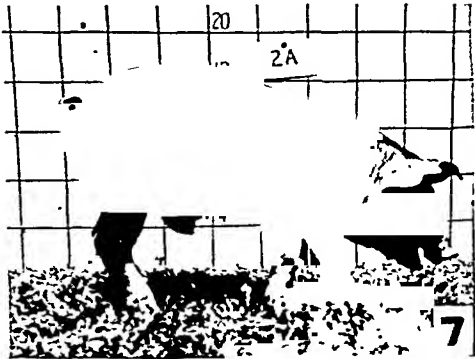
Figures 1 and 2 represent pigs on the 0.0% choline diet, whereas the pigs represented by figures 3 and 4 were fed on 0.05% choline, and by figures 5 and 6 on 0.10% choline. These pigs were in the ad libitum feeding test.



## PLATE 2

### EXPLANATION OF FIGURES

The figures on the left of the plate, viz., 7, 9, and 11, are photographs of pigs on the 0.05% choline level, whereas those on the right are their pair mates which were fed 0.10% choline. These pigs are typical of the pair-fed test.



### PLATE 3

#### EXPLANATION OF FIGURES

Photomicrographs of liver sections of pigs at termination of experiment (8 weeks). Magnification  $\times 225$ . All sections were made by paraffin method and stained with Harris's hematoxylin and Orange G.

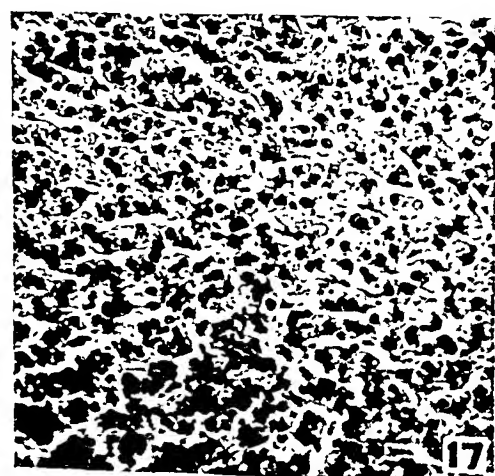
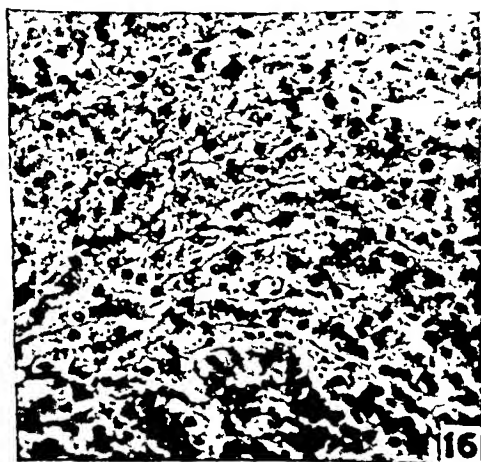
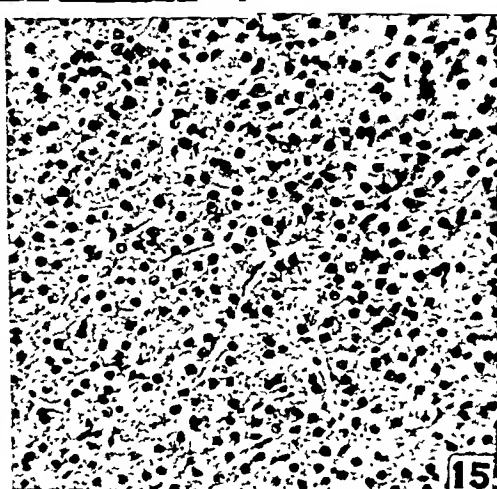
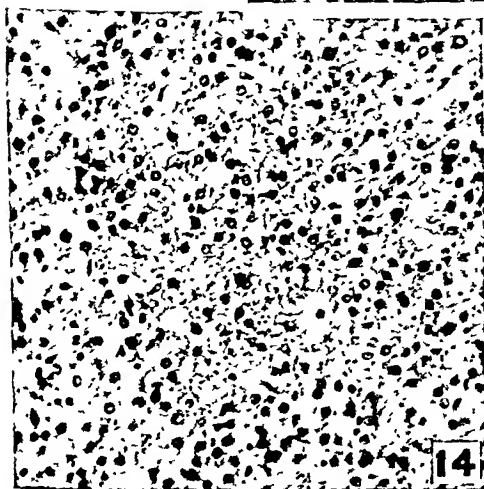
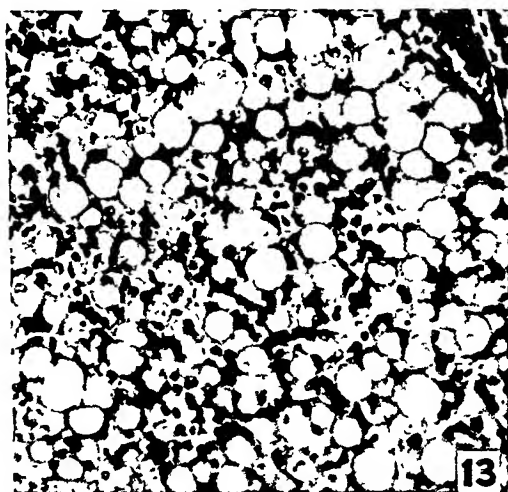
13 Liver from pig on 0.0% level of choline (see fig. 2, plate 1).

14 Liver from pig on 0.05% level of choline (see figs. 3 and 4, plate 1). Note small amount of fatty infiltration.

15 Liver from pig on 0.10% level of choline (see figs. 5 and 6, plate 1). Note absence of abnormal fatty infiltration.

16 Liver from pig on 0.05% level of choline from paired feeding test (see figs. 7, 9, and 11, plate 2). Note small amount of fatty infiltration.

17 Liver from pig on 0.10% level of choline from paired feeding test (see figs. 8, 10 and 12, plate 2).



## PLATE 4

### EXPLANATION OF FIGURES

Photomicrographs of kidney sections of pigs at termination of experiment (8 weeks). Magnification  $\times 75$ . All sections were made by paraffin method and stained with Harris's hematoxylin and Orange G.

18 Kidney from pig on 0.0% level of choline. Note extensive pathology of glomeruli and tubules.

19 Kidney from pig on 0.05% level of choline. Note some normal and some pathological glomeruli and tubules.

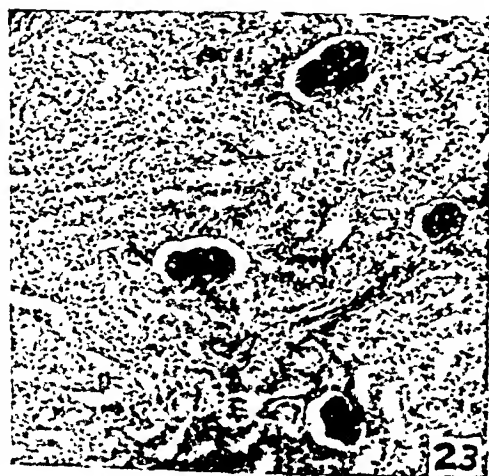
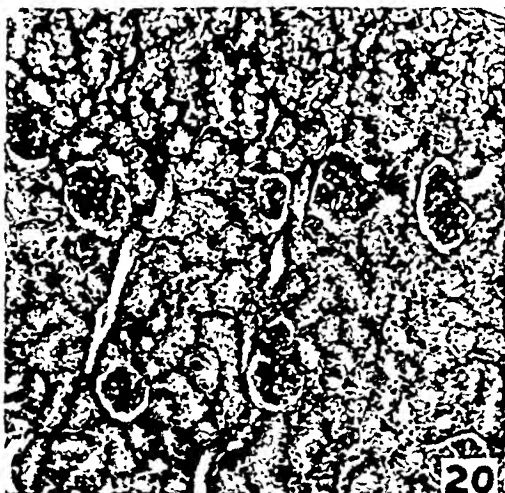
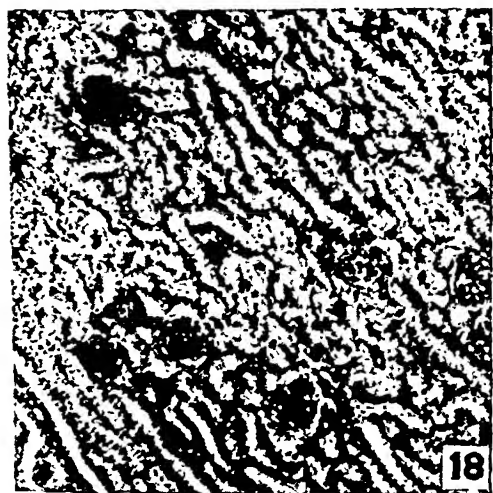
20 Kidney from pig on 0.10% level of choline. Note absence of pathological condition.

21 Kidney from pig of same age fed on an adequate farm ration. Note similarity to figure 20.

22 Kidney from pig on 0.05% level of choline from paired feeding test. Note same condition as found in figure 18.

23 Kidney from pig on 0.10% level of choline from paired feeding test. Note normal condition and similarity to figures 20 and 21.

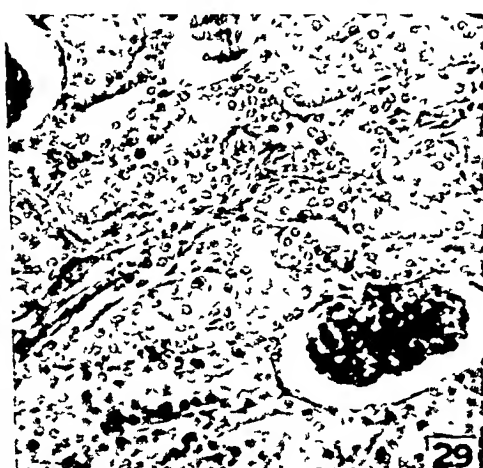
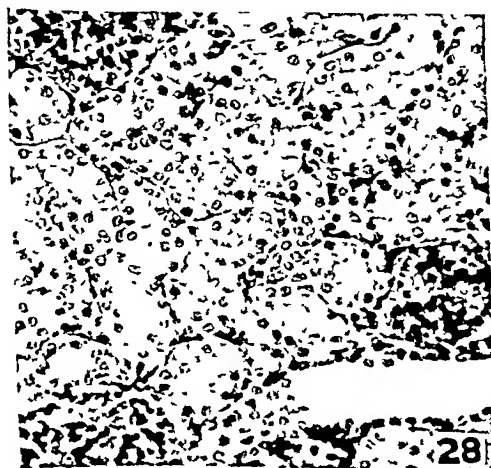
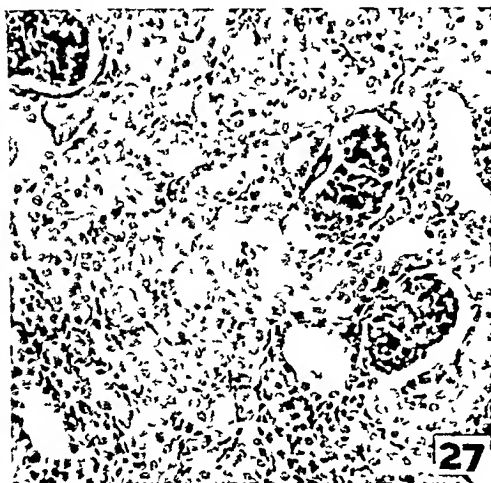
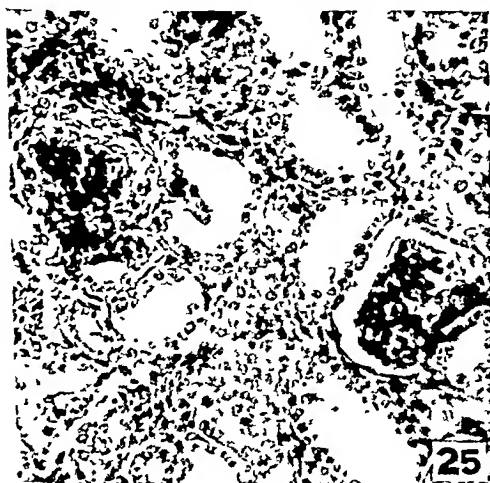
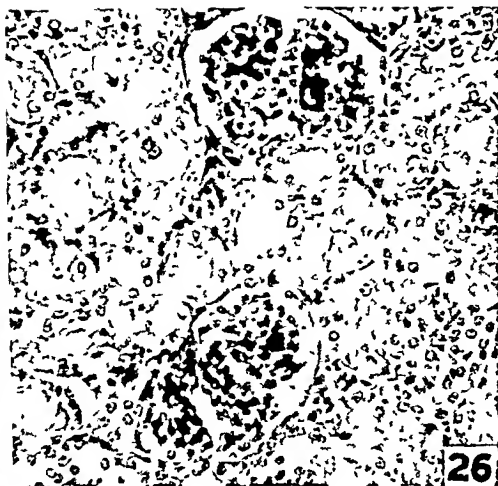
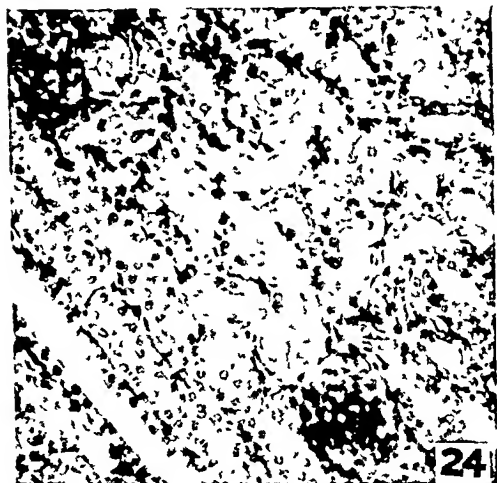




## PLATE 5

### EXPLANATION OF FIGURES

Photomicrographs of same kidney sections as shown in plate 4. Higher magnification  $\times 225$  has been used to show greater glomerular and tubular detail.





THE BIOLOGICAL VALUE OF CORN AND WHEAT  
PROTEINS IN THE MALE INFANT, WITH A  
NOTE ON THE UTILIZATION OF  
D-TRYPTOPHAN <sup>1</sup>

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During the past two years we have made measurements of the food value of some vegetable proteins in the infant. We felt this effort worthwhile for two reasons: (a) vegetable protein diets might prove useful in feeding infants allergic to animal protein diets; and (b) extensive use of vegetable protein diets may soon be necessary if the gloomy predictions of the agronomists regarding the failure of future food production to keep pace with population growth are ultimately realized. Of course, the use of vegetable protein milks (soybean, millet and cornmeal) for infant feeding has long been practiced empirically in many lands.

In this investigation, as in the previous animal tissue study from this laboratory (Albanese and associates, '48), we chose to use isolated protein fractions of wheat and corn supplemented with appropriate amounts of lysine and tryptophan rather than the whole products, to avoid gross contamination of the diet by unknown constituents or chemical structures. The nutritive characteristics of these diets are compared with

<sup>1</sup>The work described in this report was supported by grants from the Biochemical Division of the Interchemical Corporation and the Office of Naval Research.

those of diets in which the protein component was supplied by whole casein, or a tryptophan- and cystine-reinforced acid hydrolysate of casein, or evaporated cow's milk.

Quite apart from the main purpose of this report, some pertinent observations are also related on the value of DL-tryptophan as a substitute for L-tryptophan in reinforcing tryptophan-deficient diets.

TABLE 1  
*Composition of casein and zein diets*  
(Daily ration per kilogram of body weight)

DIETS	CASEIN	ZEIN <sup>1</sup>	CTH-2
	gm	gm	gm
Protein	3.5	3.2	0
Acid hydrolyzed casein	0	0	3.4 <sup>2</sup>
L-Lysine (Interchemical)	0	0.2	0
DL-Tryptophan (Merck)	0	0.1	0.1
L-Cystine (Merck)	0	0	0.4
Crisco	4.0	4.0	4.0
Dextrimaltose (special) <sup>3</sup>	12.0	12.0	12.0
Vitamin B mixture <sup>4</sup>	0.079	0.079	0.079
Salt mixture <sup>5</sup>	1.6	1.6	1.6

<sup>1</sup> Kindly supplied to us by the Corn Products Refining Company as Mazein.

<sup>2</sup> Protein = N  $\times$  6.25.

<sup>3</sup> A vitamin-free preparation kindly supplied us by Dr. Warren M. Cox, Jr., of Mead Johnson and Company.

<sup>4</sup> This mixture had the following composition, which was calculated to supply daily the same quantities as a quart of fresh cow's milk (measured in mg): thiamine, 0.4; nicotinic acid, 0.9; pantothenic acid, 3.5; pyridoxine, 0.7; inositol, 180; *p*-aminobenzoic acid, 0.5; choline, 147; riboflavin, 170.

<sup>5</sup> The salt mixture employed had the following composition (measured in gm): FeSO<sub>4</sub>, 0.9, NaCl 6, calcium gluconate 48, Ca(OH)<sub>2</sub> 12, KH<sub>2</sub>PO<sub>4</sub> 7, KCl 6, MgO 0.1.

## EXPERIMENTAL

### *Diets and procedures*

The composition of the diets employed in the first and second experiments is shown in tables 1 and 2, respectively. The level of dietary protein, i.e., 3.2 to 3.5 gm/kg of body weight, was determined by an evaluation of the available data in the

literature (Albanese, '47) and our own experience as an intake required for optimum N retention. As in previous studies, these were given in 5 daily feedings and were supplemented daily with 50 mg of ascorbic acid and 15 drops of oleum percomorphum. The composition and preparation of the diets shown in table 1 differed in several respects from

TABLE 2

*Composition of corn and wheat gluten diets  
(All diets fed at the rate of 100 Cal. and 3.5 gm of protein per kilogram  
of body weight)*

INGREDIENT	D I E T	
	Corn gluten	Wheat gluten
	%	%
Wheat gluten <sup>1</sup>	0.00	3.34
Corn gluten <sup>2</sup>	3.25	0.00
L-Tryptophan	0.04	0.00
L-Lysine	0.21	0.16
Brewers' yeast	1.00	1.00
Olive oil	4.00	4.00
Dextrimaltose no. 2	9.60	9.60
Arrowroot starch	2.30	2.30
Salt mixture <sup>3</sup>	1.60	1.60
Water	78.00	78.00
Estimated lysine content (mg/100 gm)	240.00	240.00

<sup>1</sup> Kindly supplied to us by the Biochemical Division of the Interchemical Corporation as Product G.

<sup>2</sup> Kindly supplied to us by the Corn Products Refining Company as Product no. 3323.

<sup>3</sup> The salt mixture employed had the following composition (measured in gm): FeSO<sub>4</sub> 0.9, NaCl 6, calcium gluconate 48, Ca(OH)<sub>2</sub> 12, KH<sub>2</sub>PO<sub>4</sub> 7, KCl 6, MgO 0.1.

those which we customarily employ for these bioassays. Instead of brewers' yeast a synthetic mixture of the commercially available B vitamins was used and the diet components were mixed with water to a cereal-like consistency and spoon-fed, in contrast to the milk-like consistency of our usual diets (table 2), which are bottle-fed.

The diet periods were of 7 days' duration and consecutive, but collections of excreta were omitted on week-ends. The

subjects were partially immobilized by specially designed abdominal supports which also held the urinary adapters in place. Daily 24-hour urine specimens were collected in bottles containing 10 ml of 15% (by volume) HCl and 1 ml of 10% alcoholic thymol. The feces were collected in 19 cm porcelain evaporating dishes which were held in place by a properly shaped excavation in the mattress, and the daily stools were

TABLE 3

*Comparison of biological value of zein, casein and acid hydrolyzed casein formulae in the normal male infant*  
(All results given as daily averages of 7-day periods)

SUBJECT	DIET	TOTAL N INPUT	DAILY BODY WEIGHT CHANGE	NITROGEN RETENTION	TOTAL PLASMA PROTEIN	DAILY FECAL N
		<i>gm</i>	<i>gm</i>	<i>mg/kg</i>	<i>gm %</i>	<i>gm</i>
S.A. 7 months 6,230 gm	Casein	3.54	+ 12	174	6.90	0.14
	Zein	3.54	— 7	45	7.65	1.97
	Zein	3.54	+ 5	— 19	5.70	2.21
	Casein	3.54	+ 12	194	5.99	0.17
	Casein	3.72	+ 12	173	5.92	0.21
	CTH-2	4.12	— 8	144	6.41	0.21
	CTH-2	4.12	— 28	137	6.90	0.16
	Casein	3.90	+ 24	178	7.38	0.15
	Zein	3.90	+ 23	80	7.81	1.45
G.O. 6 months 6,965 gm	Zein	3.90	— 17	56	6.13	1.35
	Casein	3.90	+ 23	132	5.53	0.33
	Casein	3.90	+ 17	136	4.25	0.20
	CTH-2	4.25	+ 17	58	7.12	0.27
	CTH-2	4.25	+ 10	64	7.16	0.23

accumulated under refrigeration for each period in wide-mouth jars containing 200 ml of 70% alcohol. The subjects were weighed daily during the course of the experiment.

The data on nitrogen retention were calculated from the results of nitrogen determinations on the 24-hour urine collections, analyses of the pooled feces from each period, and computations of the daily N intake based on food consumption records and the determined N content of the diets.



Blood samples were collected over lithium oxalate by vena puncture on the last day of each diet period. The hemoglobin concentration of these specimens was determined colorimetrically in the Klett-Summerson photoelectric colorimeter. The total plasma proteins, albumin, globulin and non-protein N were determined by the procedure described by some of the present authors (Albanese, Irby and Saur, '46).

## RESULTS AND DISCUSSION

### *First experiment*

The most striking feature of this study is the finding with respect to the very poor biological value of the supplemented zein diet (table 3). The nutritional inadequacy of zein for the rat has been known since the work of Osborne and Mendel (Mendel, '15), but they found that the deficiencies could be corrected by the addition of tryptophan and lysine to the diet. The increase in fecal N output (some 50% or more of the intake) observed during the zein diet period indicates that this apparent species difference can be ascribed primarily to poor assimilation of zein by the infant. Isolation experiments disclosed that the extra fecal N occurring in the stools collected during this diet period was in the form of whole zein, which could be readily isolated from the stools. *In vitro* experiments disclosed that in 6 hours commercial digestive enzymes, pancreatin and trypsin derived from hog tissues, hydrolyzed zein to about one-third the amino N level attained with casein under comparable conditions. From these evidences it appears that the poor nutritional quality of zein in the infant is to be attributed primarily to its poor digestibility. It should be emphasized, however, that the poor digestibility of the commercial zein must have been caused by the processing to which it was submitted. It is not a characteristic property of native zein.

Turning our attention to the other diet periods of this experiment, we observe that the N-retention and weight change values attained on the whole casein diet fall well within the

ranges previously found for the enzymatic digests of casein, lactalbumin, beef muscle and evaporated milk diets (Albanese and co-workers, '47). These were attained, it should be noted, in the absence of a cystine supplement. Indeed, the nutritional value of this diet was superior to that of the CTH-2 which was supplemented with L-cystine.

The poor nutritional quality manifested by the CTH-2 diet employed in this experiment is to be contrasted with that of the CTH diet tested in the earlier study. These diets differed in several respects: (a) in the present diet (CTH-2) a mixture of the synthetic B vitamins was substituted for the brewers' yeast of the CTH diet; (b) as noted above, the diets had a different physical consistency; and (c) the shortage of L-tryptophan forced us to use DL-tryptophan (in double quantity) in the preparation of diet CTH-2. Inasmuch as the subjects performed well on the casein diet, it is fair to assume that the poor quality of diet CTH-2 could not be attributed to a B vitamin deficiency or to poor assimilation because of its consistency. It remains, therefore, to question the use of DL-tryptophan as a physiological substitute for L-tryptophan. Evidence suggesting that this substitution may have untoward nutritional effects can be derived from the atypical brilliant red coloration obtained with the application of the Albanese-Frankston procedure ('45) for the determination of urinary tryptophan to the urine collected during the zein and CTH diet periods. This anomaly subsided almost immediately with the feeding of the casein or evaporated milk diets. Concomitantly, abnormally high values were obtained with the Shaw-McFarlane method (Berg and Rohse, '47) which were not apparently due to the excretion of D-tryptophan. The urinary tryptophan values as obtained by the microbiological procedure (Greene and Black, '44) were totally unaffected by the dietary changes. Oddly enough, negative tests were obtained with the urines of the zein and CTH period for the aberrant metabolite which we reported to be excreted by adults after the administration of DL-tryptophan (Albanese and Frankston, '44). This may be due to a differ-

ence in the relative amounts of tryptophan given, or to some differences in metabolic response between the adult and infant. It is obvious that further and more detailed studies are required for a complete evaluation of the utilization of D-tryptophan by man.

TABLE 4

*Comparison of biological value of evaporated milk and corn gluten formulae in the normal male infant*  
(All results given as daily averages of 7-day periods)

SUBJECT	DIET	TOTAL N INPUT	DAILY BODY WEIGHT CHANGE	NITROGEN RETENTION	TOTAL PLASMA PROTEIN	DAILY FECAL N
		gm	gm	mg/kg	gm %	gm
T.R. 4.5 months 6,370 gm	EM	3.31	+ 17	140	6.70	0.60
	CG	3.65	— 59	135	7.43	0.55
	CG	3.68	— 34	26.5	6.80	1.47 <sup>1</sup>
	CG	3.53	— 5	65.5	7.50	0.51
	EM	3.45	+ 23	129	6.26	0.46
A.J. 6.0 months 6,837 gm	EM	3.88	+ 23	139	7.20	0.34
	CG	3.23	0	98	7.00	0.38
	CG	3.81	— 66	100	7.65	0.24
	CG	3.88	+ 3	48	7.68	0.72
	CG	3.90	+ 23	81	7.00	0.43
C.L.Y. 6.3 months 7,107 gm	EM	3.50	+ 17	121	7.11	0.57
	EM	4.00	+ 23	133	6.10	0.61
	CG	3.98	— 27	116	6.53	0.53
	CG	4.11	+ 15	60	6.10	0.53
	CG	4.17	+ 12	73	6.62	0.73
	EM	3.93	+ 12	132	6.10	0.52

<sup>1</sup> This high fecal N was caused by a diarrhea of cryptic origin.

### *Second experiment*

Returning to the principal theme of this report, a nutritional evaluation of corn and wheat proteins, we next considered the possibility that the inferior biological value of the reinforced zein diet might be due to chemical changes induced in the zein during manufacture. It occurred to us, therefore, to assay corn gluten, a product which is subjected to less vigorous chemical treatment than zein. On the basis

of the experience already discussed, L-tryptophan instead of the racemate was used to correct the tryptophan deficiency of the protein. The results of this study, which are shown in table 4, disclose that this product, like zein, exhibited poor nutritional qualities. However, examination of the fecal N data discloses that this characteristic cannot be ascribed, as in the case of zein, entirely to poor digestibility. In the light of recent observations one is tempted to suggest that it may be due to an amino acid imbalance, a subject which has been

TABLE 5

*Comparison of biological value of lysine-fortified gluten (WG) and evaporated milk (EM) formulae in the normal male infant*  
(All results given as daily averages of 7-day periods)

SUBJECT	DIET	TOTAL N INPUT	DAILY BODY WEIGHT CHANGE	NITROGEN RETENTION	TOTAL PLASMA PROTEIN	DAILY FECAL N
		gm	gm	mg/kg	gm %	gm
D.J. 3 months 4,06½ gm	EM	2.81	+ 28	200	6.63	0.40
	EM	2.75	+ 28	182	5.81	1.35
	WG	2.74	+ 19	188	5.65	0.36
	WG	2.94	+ 24	188	6.21	0.34
J.A. 4 months 3,369 gm	EM	2.45	+ 37	162	6.63	0.40
	EM	2.53	+ 28	165	5.81	0.47
	WG	2.56	+ 22	179	5.65	0.42
	WG	2.67	+ 26	190	6.21	0.42
S.M. 3 months 3,230 gm	EM	2.46	+ 34	215	6.42	0.32
	EM	2.49	+ 23	222	5.96	0.29
	WG	2.35	+ 25	196	6.21	0.31
	WG	2.45	+ 28	195	6.18	0.57

recently reviewed by Elvehjem and Krehl ('47). A comparison of the amino acid composition of corn gluten and casein shows the principal difference to lie in the high leucine content of corn gluten (corn gluten 25%, casein 12%, Block and Bolling, '45).

Although this hypothesis is founded on surmise, supporting evidence can be adduced from the results of the bioassay of the lysine-reinforced wheat gluten diet, which show that diet WG (containing 12% leucine) supports nutritional

states in the infant comparable to those supported by the evaporated milk diet. Doubtlessly, numerous other factors may be responsible for the nutritional differences between the corn and wheat gluten diets, but the disparity in leucine content of the two products is an obvious variant. Because of the relative abundance and low cost of corn gluten, it would seem economically worthwhile to explore means of remedying its nutritional defects so that more complete utilization could be realized.

#### SUMMARY

It has been found that the nitrogen retention and weight gain of infants maintained on a synthetic diet in which tryptophan- and lysine-supplemented commercial zein constituted the principal source of nitrogen were inferior, by reason of the poor digestibility of the zein, to those obtained on a casein diet fed at the same fluid, caloric distribution and nitrogen levels. A diet in which tryptophan- and lysine-reinforced corn gluten provided the major source of protein also proved to be nutritionally inadequate for the infant. In contrast, a lysine-enriched wheat gluten diet supported a nutritional state in the infant comparable to that afforded by an evaporated milk formula. Evidence, albeit inconclusive, is presented which suggests that the inclusion of D-tryptophan in the diet may have an untoward nutritional effect.

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# THE AVAILABILITY OF VITAMINS FROM YEASTS

## V. DIFFERENCES IN THE INFLUENCE OF LIVE YEAST ON THE ABSORPTION OF PURE THIAMINE HYDROCHLORIDE, PURE RIBOFLAVIN AND NITROGEN BY HUMAN SUBJECTS, AND THE EFFECT OF DISTRIBUTION OF THE VITAMIN DOSES <sup>1</sup>

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Earlier experiments established that when live bakers' yeast is ingested by human subjects, not only the yeast thiamine but also food thiamine simultaneously consumed fails to be returned in the urine in normal amounts, presumably through failure of absorption (Parsons et al., '45). Reports by Bird et al. ('46) and Mims et al. ('47) of an inhibitor of the action of the vitamin B<sub>6</sub> conjugase in crude yeast extracts suggested experiments to determine whether there might be a similar enzyme inhibitor in yeast which interferes with the availability of the thiamine from food in the diet and from fresh yeast, a variable part of which is in the combined form. This possibility appeared at first to be excluded, as it had

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already been shown (Hochberg et al., '45) that thiamine ingested in a certain sample of bakers' yeast in which 83% was in the free form was only 17% available to human subjects, and hence the unavailability does not depend necessarily on the occurrence of the thiamine in a combined form when ingested. However, inasmuch as yeast cells can be influenced to alter the form of their contained thiamine, as for example when certain conditions of drying induce a shift of some of the combined yeast thiamine into the free form (Melnick and Field, '39), one could not rule out arbitrarily the possibility that contact with digestive juices and other conditions in the intestinal tract might in some way induce the live yeast cell to convert its free thiamine to a form in which its release for absorption might be blocked by a yeast inhibitor.

Such an inhibitor then could conceivably account for the low availability of the thiamine, not only in the yeast of Hochberg et al. ('45) but also in the basal diet of Kingsley and Parsons ('47) in the presence of live yeast. In the latter case it would be assumed that only combined thiamine in the diet was being interfered with, and a mode of its encountering the hypothetical inhibitor in yeast could be postulated. Hence, in the present experiment, it was planned that so large a proportion of the thiamine intake accompanying the fresh yeast supplement should be in the free form that any interference with its absorption, if this were clearly shown, would require a modification of the hypothesis of an inhibitor stated above. Assays for riboflavin and nitrogen were planned in order to extend the basis for interpreting the nature of the influence of fresh yeast in the diet.

#### EXPERIMENTAL

• The method of study was similar to that reported by Price et al. ('47), i.e., a basal diet<sup>4</sup> containing about 0.63 mg of thiamine and 3.7 to 4.2 mg of riboflavin per day (determined by weekly assay for thiamine by the thiachrome method of Hen-

<sup>4</sup>We are indebted to the Pineapple Research Institute of Hawaii for the gift of the specially packed crushed pineapple.



nessy, '47, and for riboflavin by the fluorometric method of Conner and Straub, '41) was ingested by normal college women over a 4-day period. The single test-day was preceded and followed by periods on the basal diet alone. As this short human bioassay was not suitable for determining nitrogen balances, the study of the availability of the protein of fresh yeast F was carried out with a diet squad maintained on different intakes for periods of three to 10 days (Ness et al., '46). Nitrogen was determined by macro-Kjeldahl assay.

Supplements included 5 mg of thiamine hydrochloride, 3 mg of riboflavin, 100 or 150 gm of a regular fresh live yeast prepared for the use of bakers (yeast F) and containing 4 to 7  $\mu$ g of thiamine and 14  $\mu$ g of riboflavin per gram (moist basis), and a preparation of non-viable yeast F'. The yeast was killed by suspension in 70% ethanol for 48 hours and subsequent reduction to dryness before a fan in a darkened room. The viability of this alcohol-dried yeast was determined by plating various dilutions of the yeast suspension on a nutrient medium, and was found to be negligible.

The criterion of availability of thiamine and riboflavin from the yeast and from the supplements of pure vitamins used in this study was their urinary return as compared with that from positive control doses of 5 mg thiamine hydrochloride and 3 mg riboflavin given with the basal diet above but without the presence of yeast.

## RESULTS

### *Results on thiamine*

When free thiamine was given as the sole test dose with the regular meals, the urinary return was 22.4%. When free thiamine and fresh yeast were ingested together with meals, the urinary thiamine excretion was decreased to an average of 0.2% of the test dose (table 1). Two subjects of the 5 showed a decrease in urinary thiamine output below that on the basal diet alone. It can be concluded, then, that not only combined food thiamine is interfered with by fresh yeast ingestion, but

TABLE 1

*Effect of presence of live yeast in diet and of time of intake of doses of thiamine and riboflavin on urinary return of vitamins*

SUBJECTS	PERCENTAGE RETURN OF THIAMINE <sup>1</sup>		PERCENTAGE RETURN OF RIBOFLAVIN <sup>1</sup>		
	5 mg thiamine HCl alone		3 mg riboflavin alone		3 mg riboflavin with fresh yeast <sup>1</sup>
	Between meals	With meals	Between meals	With meals	
FG	10.3	19.9	0	62	68
ZB	5.8	25.6	0 <sup>2</sup>	54	53
MP	15.8	28.6	0	53	64
PH	9.9	20.5	0.8	64	31
BC	20.6	17.5	0.3	48	64
Average	11.5	22.4	0.2	57	56

<sup>1</sup> Formula and method explained by Kingsley and Parsons ('47). When fresh yeast was included with the pure vitamin supplements, the total dose of thiamine was 5.4 mg and of riboflavin 4.4 mg.

<sup>2</sup> Lag day not carried out.

that viable yeast similarly affects the availability of thiamine hydrochloride ingested in pure solution.

It was desired to determine more clearly the effect of fresh yeast on the pure vitamin alone, without the presence of food. Hence, the supplements were given without the accompaniment of food and as far removed from meals as was convenient for the administration of doses in the laboratory. All subjects ingested 100 gm of yeast per test-day, divided in the following manner: 15 gm of yeast with each meal, and 55 gm of yeast plus 5 mg thiamine hydrochloride and 3 mg riboflavin in one dose between meals.

Urinary thiamine returns after the ingestion of free thiamine, plus that combined in fresh yeast taken in three doses with meals, averaged 0.2%. The average thiamine return from this same intake of the pure vitamin when ingested at one time, between meals with the fresh yeast, was 5.7% for 8 subjects, with a range of from 2.1% to 9.3%. This difference may be due not so much to the timing of the vitamin doses as to the allocation of too large a proportion of the yeast dose to the between-meal feeding of the pure vitamin, leaving too small an amount (15 gm) to accompany the meals and thus perhaps permitting more of the food thiamine to be absorbed by the digestive tract in the latter plan of feeding than in the former. In either case the simultaneous ingestion of fresh yeast with the thiamine supplement caused an unmistakable and striking depression of the urinary return below the level expected from ingestion of the vitamin alone.

It seemed desirable to test specifically the effects of the two methods of distribution of doses of the vitamins themselves without the accompaniment of yeast. The urinary returns of pure thiamine hydrochloride ingested as a single dose between meals averaged only 11.5% for 6 subjects (table 1), as compared with an average return of 22.4% for the same subjects ingesting the vitamin supplement with meals. Repeating this test with available thiamine in the form of dead dried yeast, distributed in the two ways, the average return for between-meal ingestion of the dose was only 13%, in contrast

to an average of 25.3% when the supplement was taken with the meals, thus closely conforming to the results obtained with the pure vitamin.

Melnick et al. ('39) observed a similar order of difference in urinary excretion of thiamine; the difference was greater when the positive control dose was taken with the heaviest meal of the day than when the subjects were in a post-absorptive and fasting state "... 12 hours after the last meal and 3 hours prior to breakfast." The same workers ('41) suggested that this trend of decreased urinary excretion following a test dose of thiamine hydrochloride may be due to one or both of two factors: the first postulation was that thiamine destruction is due to its instability in a relatively more alkaline bile and pancreatic juice in the post-absorptive state, when gastric secretion is at a minimum. Investigating the fate of thiamine *in vitro* in various digestive secretions, they found appreciable destruction ranging from 50% to 90% at pH 8.0 to 8.5 in the presence of bile or pancreatic juice. The second postulation was the possible absorption of the vitamin from the stomach during a relatively longer retention in that organ when the presence of food prolonged the emptying time.

The same contrast between returns of urinary thiamine which Melnick et al. ('39) demonstrated when thiamine was fed with the meal and 12 hours later, respectively, was almost exactly duplicated in the present experiments, when the interval after the meal was only two to two and one-half hours. Hence, the hypothesis of Melnick and his associates that this contrast is necessarily attributable to differences in the effects of a full stomach versus an empty, alkaline stomach is shown not to be tenable.

### *Results on riboflavin*

Urinary riboflavin excretions following a 3 mg positive control dose of pure riboflavin taken with meals ranged from 31% to 77% for 13 subject-periods, averaging 54%. When fresh yeast containing 1.4 mg riboflavin per 100 gm dose

was ingested with the 3 mg riboflavin at meals, 5 subjects had an average return of 41% of the total dose, with a range of 34% to 55% (table 1). The availability of riboflavin from 100 or 150 gm supplements of the fresh yeast alone had been ascertained with 8 subjects; there was an average return of only 16% of the riboflavin content of the yeast, thus confirming earlier observations by Price et al. ('47). Fresh yeast contributed 1.4 mg or approximately one-third of the riboflavin of the total test dose when it was taken together with 3 mg pure riboflavin. Hence, considering the relative unavailability of riboflavin from fresh yeast and the range of individual variations in response to the positive control doses, it does not appear that live yeast interfered with the absorption of pure riboflavin when taken with meals.

In studies of supplements ingested between meals, 32% to 77% of the riboflavin from a 3 mg positive control dose was excreted in 17 subject-periods; the average was 57%. Six subjects were given the 3 mg pure riboflavin plus fresh yeast between meals. Returns ranged from 35% to 53%, averaging 46%. Therefore, contrary to results with thiamine, there was no striking difference in riboflavin returns whether the doses were taken with meals or between meals. Similar results were obtained when dead dried yeast, shown previously by Price, Marquette and Parsons ('47) to contain available riboflavin, was the supplement together with the pure riboflavin. The average return from between-meal ingestion of the dose was 52% for 10 subjects, and 53% when taken with meals by three subjects.

### *Results on nitrogen*

Evidence is presented in table 2 that the protein of the fresh yeast cell is well absorbed by the human digestive tract. After a 6-day period on the basal diet alone, supplementation with 150 gm of fresh yeast F in a 6- or 10-day period for 6 subjects led to an average recovery in the urine of 80% of the 3.72 gm of nitrogen furnished by the yeast. This may be

TABLE 2

*Comparison of urinary and fecal thiamine output with urinary and fecal nitrogen output on a basal diet with and without fresh bakers' yeast*

Type <sup>1</sup>	PERIOD	Length days	NITROGEN ELIMINATIONS				GM/DAY		THIAMINE ELIMINATIONS	
			Individual subjects <sup>2</sup>				AVE.		Avg. $\mu$ g/day <sup>2</sup>	
			Urine	Feces	Urine	Feces	Urine	Feces	Urine	Feces
Basal		6	9.70	1.42	9.78	1.05	9.69	1.27	312	680
Basal plus 150 gm fresh yeast F		6	12.98	1.55	12.46	1.52	12.75	1.64	98	1089
Basal		3	10.42	1.32	10.37	1.27	10.73	1.49	226	582
Basal		6	9.04	9.55	10.45	9.68	9.68	332		
Basal plus 150 gm fresh yeast F		10	13.07	11.66	13.0	12.58	12.58	68		
Basal		3	9.12	11.0	9.67	9.93	9.93	140		

<sup>1</sup> The basal diet contained 11.2 gm nitrogen; the 150 gm yeast F, 3.72 gm nitrogen.

<sup>2</sup> The two diet squads comprised three subjects each; fecal values are available for one squad only.

TABLE 2  
*Comparison of urinary and fecal thiamine output with urinary and fecal nitrogen output on a basal diet with and without fresh bakers' yeast*

Type <sup>1</sup>	Length days	NITROGEN ELIMINATIONS						GN/DAY		THIAMINE ELIMINATIONS	
		Individual subjects <sup>2</sup>						Ave.		Ave. µg/day <sup>2</sup>	
		Urine	Feces	Urine	Feces	Urine	Feces	Urine	Feces	Urine	Feces
Basal	6	9.70	1.42	9.78	1.05	9.69	1.27	9.72	1.37	312	680
Basal plus 150 gm fresh yeast F	6	12.98	1.55	12.46	1.52	12.75	1.64	12.73	1.57	98	1089
Basal	3	10.42	1.32	10.37	1.27	10.73	1.49	10.51	1.36	226	582
Basal	6	9.04		9.55		10.45		9.68		332	
Basal plus 150 gm fresh yeast F	10	13.07		11.66		13.0		12.58		68	
Basal	3	9.12		11.0		9.67		9.93		140	

<sup>1</sup> The basal diet contained 11.2 gm nitrogen; the 150 gm yeast F, 3.72 gm nitrogen.

<sup>2</sup> The two diet squads comprised three subjects each; fecal values are available for one squad only.

the present experiment, in which it has been shown that pure thiamine hydrochloride is influenced in the digestive tract in the same manner as is food thiamine. It would appear to be more reasonable to postulate that the phenomenon involves an uptake of food thiamine by the live yeast cells analogous to the thiamine uptake from wort in the making of beer. Riboflavin is known not to be taken up appreciably by yeast from surrounding media such as wort, which would be in harmony with the observation that only the riboflavin of the live yeast cell, not that of the food or in pure form in the presence of live yeast, is relatively unavailable to the human digestive tract.

On the other hand, this hypothesis without further elaboration does not account for the strikingly higher utilization of nitrogen than of thiamine or of riboflavin from the fresh yeast cell. The true explanation is probably a more complicated one than either of the above hypotheses suggests and clarification must await further observations.

#### SUMMARY

A higher percentage of a dose of thiamine hydrochloride was returned in the urine of human subjects when the supplement was ingested with meals rather than between meals. The urinary return of riboflavin was not similarly influenced.

Pure thiamine hydrochloride ingested with fresh bakers' yeast failed to be returned in the urine, presumably through almost complete failure of absorption by the digestive tract. This was the same effect as that observed previously in respect to food thiamine ingested with live yeast. Hence, the "interference" by fresh yeast is not attributable per se to the effect of an anti-enzyme in the digestive tract.

In the same experiment live yeast interfered less with riboflavin than with thiamine in that only the riboflavin of the live yeast cell, not that in the food or in pure form, appeared to be withheld from absorption; nitrogen was least affected, inasmuch as an average of 80% of the nitrogen of the yeast cell itself was returned in the urine. That these



differences in excretion were actually due to differences in absorption tended to be borne out by fecal assays.

These observations make necessary some further elaboration of the hypothesis that food thiamine is interfered with by live yeast because of competition between the absorbing membranes of the live yeast cell and of the digestive tract, with a resulting retention of the absorbed thiamine within the yeast cell.

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# THE DISTRIBUTION OF THIAMINE IN THE EMBRYONATED HEN EGG

## I. THE CONTENT OF THE WHOLE EMBRYO <sup>1</sup>

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### ONE FIGURE

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The thiamine content of the egg of the domestic fowl, as determined by the macro fermentation method, has been reported by this laboratory to remain essentially unchanged during incubation (Scrimshaw et al., '44). Before this work was completed, Westenbrink and Van Leer ('41) reported that most of the thiamine originally present in the egg fails to appear in the embryo. Their procedure differed from ours since they followed separately the drop of thiamine in the yolk and its increase in the developing embryo. Little is known of the role of thiamine in embryonic development, and the question of its utilization in the morphogenesis and maintenance of the chick embryo is of considerable interest. Therefore, it seemed necessary to investigate this apparent disagreement concerning the fate of the thiamine originally present in the egg. Accordingly, the procedure of Westenbrink and Van Leer of studying separately the thiamine in the embryo and in the extra-embryonic portion of the egg was followed in the present study.

The results of Westenbrink and Van Leer were not confirmed, although a small but significant loss of thiamine in

<sup>1</sup> Aided by a grant from Swift and Co., Chicago, Illinois.

the whole egg during development has now been demonstrated by using a larger number of eggs per sample and improved methods of preparation. This was obscured in the earlier investigation by the greater variation in the values and the failure to correct for the small total weight loss which occurs during incubation. This small drop in the total thiamine content is believed to represent the thiamine required for metabolic processes during development. However, in general, the assays carried out on these materials by the macro fermentation method and described below confirm the results previously published from this laboratory. Various bases for the above discrepancy are explored and a partial explanation is suggested.

#### MATERIAL AND METHODS

Ten eggs<sup>2</sup> of each series were selected at random and assayed as a single sample to determine a base-line thiamine value for the incubated group. The remainder were placed immediately in a standard electric incubator with fan and kept at temperatures of 38° to 41°C. and a humidity of approximately 40%. The eggs were turned twice a day.

Ten eggs were removed at the intervals listed and made up as a single sample. The eggs were weighed and then broken. The contents were then either weighed as a whole or separated so that the embryo and the remainder could be recorded separately. The "remainder" ordinarily consisted of all of the egg contents except the embryo and therefore included the yolk, allantois, amnion, and so forth. In a few specified cases, the eggs were divided into embryo, yolk, and remainder. These samples were then mixed in a Waring Blendor and diluted to a convenient volume. In the case of larger samples, an aliquot was weighed into a volumetric flask and diluted. All samples were then brought to a pH of 4.00 or below by the addition of HCl and stored in a cold room at about 5°C.

<sup>2</sup> The eggs were supplied from White Leghorn hens by Roger's Farms, a nearby commercial poultry farm and hatchery.

Samples thus prepared could be stored for several weeks without change in their thiamine activity.

All assays were carried out by the modified macro fermentation method (Scrimshaw and Stewart, '44). Duplicate runs were made whenever the quantity of sample permitted. Many values were checked by two separate cleavages and preparations of a sample and two runs in duplicate. Most samples were assayed at approximately the 2  $\mu$ g level. All samples were heated for 45 min. in a boiling water bath in preparing them for assay, but no special digestion procedure was used. Autolysis at room temperature did not appear to change the thiamine content of acidified samples.

TABLE 1  
*Thiamine content of base-line samples of the series studied*

SERIES	DATE	NO. IN SAMPLE	MEAN SHELL WT.	MEAN CONTENT WT.	B <sub>1</sub> / EGG	B <sub>1</sub> / 100 gm
			gm	gm	$\mu$ g	$\mu$ g
V	10/12/44	10	6.7	50.3	48.3	96
VI	10/27/44	10	6.5	42.6	42.6	100
VII	1/4/45	20	6.7	47.6	44.3	93
VIII	1/29/45	10	7.4	51.8	37.3	72
IX	2/8/45	10	8.3	50.7	40.6	80
X	3/10/45	10	6.6	48.5	48.5	100
XI	4/12/45	10	7.3	50.6	50.6	100

#### EXPERIMENTAL RESULTS

Nine large lots of eggs were incubated during the fall of 1944 and the spring of 1945. The dates on which incubation was begun along with data on the base-line samples are shown in table 1. There is some suggestion that the lower values for series VII and VIII were associated with unusually severe winter weather at that time. The values obtained for the embryo and egg contents of the various samples are listed in table 2. When the egg and embryo thiamine values for all series are adjusted to an arbitrary base-line value of 100  $\mu$ g per 100 gm of whole egg, they can be presented together.

The average thiamine content data are shown in table 2, but the sharp increase in thiamine content of the embryo and the relatively small loss of total thiamine in the egg are best shown in figure 1, where the individual sample values are plotted. Although the error in assay of late stages is relatively high, there is still evidence for a slight drop in thiamine content. This was obscured in the earlier study by factors indicated above. The curve for the increasing thiamine con-

TABLE 2

*The thiamine concentration and total thiamine content of the developing embryo*

AGE IN DAYS	EMBRYO				REMAINDER OF EGG			
	Concentration		Content		Concentration		Content	
	Actual	Cor- rected	Actual	Cor- rected	Actual	Cor- rected	Actual	Cor- rected
	$\mu\text{g/gm}$		$\mu\text{g}$		$\mu\text{g/gm}$		$\mu\text{g}$	
0					92 <sup>1</sup>	100 <sup>1</sup>	44.8 <sup>1</sup>	50.0 <sup>1</sup>
6	45	46	0.38	0.40	108	108	50.0	48.7
10	47	48	1.45	1.31	101	102	52.0	48.4
12	58	58	3.8	3.5	120	120	47.9	44.8
13	49	50	4.8	5.0	75	77	28.5	29.7
15	75	75	10.3	9.6	97	97	31.3	29.3
16	58	58	10.6	10.6				
18	90	113	20.6	19.8	91	114	33.3	32.0
19	76	85	30.3	29.5	64	64	10.4	9.7
20					53	66	10.2	9.8
1-day chick	109		39.1		23		0.5	

<sup>1</sup> Average 7 series.

tent of the embryo shows clearly that the bulk of the thiamine in the egg is not incorporated into the embryo until relatively late in the latter's development.

The tabulation of the total thiamine content of embryonic tissues reflects primarily the relative organ masses. The concentration of thiamine per gram in the various organs is a better indication of the importance of thiamine in their metabolism. In table 2 the rising thiamine concentration of the embryo is portrayed, along with the changes in thiamine

concentration of the remainder of the egg. Due to its minute size, we did not attempt to assay the embryo before the 6th day of incubation. The thiamine concentration appears to rise in a regular fashion in the embryo. It will be noted that the highest concentration of thiamine is found in the one-day-old chicks. On the other hand, there is no important change in the concentration of thiamine in the remainder of the egg until relatively late in development. The value for the remainder

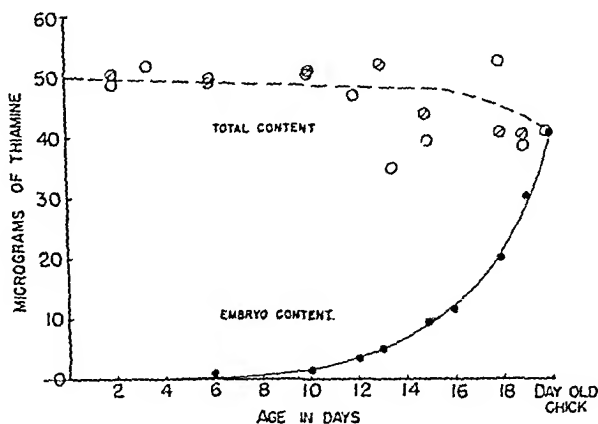


Fig. 1 The thiamine content of the whole egg and embryo during incubation. The small solid circles represent the content of the embryo at the various stages indicated. The large circles indicate the total thiamine content of the egg. The broken large circles represent values obtained by analysis of the entire egg contents. The clear large circles show the sum of the thiamine content of the embryo and non-embryo portions of the egg determined separately. All of these values have been corrected for initial variations in the thiamine concentration and weight of the eggs.

does not represent the thiamine concentration of the yolk. Although not included in the table, the initial concentration of thiamine in the yolk is approximately 300  $\mu\text{g}$  per 100 gm; in the 18-day chick it has dropped to 120  $\mu\text{g}$ ; and as "spare yolk" the day-old chick contains about 20  $\mu\text{g}$  per 100 gm. In the early stages all of the thiamine is contained in the yolk but as development proceeds the thiamine becomes widely distributed.

## DISCUSSION

As noted above, all of the thiamine originally present in the egg is to be found in the yolk (Chick and Roscoe, '29; Bethke et al., '36; Ellis et al., '33; Scrimshaw and Stewart, '44). The distribution of this substance, like that of many others, must be regulated by the living yolk membrane (Needham, '31). The work involved in maintaining these tremendous concentration gradients is probably sufficient to account for most of the metabolic activity of the unfertilized egg (0.06 cal. per hour at 19°C., Langworthy and Barott, '21). After long storage the vitality of this yolk membrane is lost and osmotic gradients tend to disappear (Straub and Hoogerduijn, '29). In studying eggs which had been stored in the cold room for 16 months, we found an average thiamine content for the white of 40  $\mu$ g per 100 gm. It is apparent that the ability of the membrane to maintain a steep thiamine gradient is also lost with age.

The loss in thiamine content of the non-embryo portion of the egg which appears to set in after the 15th day must in part represent the relatively greater drop in the volume of yolk (low thiamine content). That the yolk itself also drops in thiamine concentration is best shown by the figure for the remains of the one-day-old chick. Here the sample was obtained by dissecting out the "spare" yolk and the assay value represents yolk alone. This figure of approximately 20  $\mu$ g per 100 gm is but 7% of the original concentration. Some of this drop undoubtedly occurred during the hours of muscular activity after hatching.

In a previous report (Scrimshaw et al., '44) the thiamine concentration per 100 gm of whole egg was discussed as if it were a direct reflection of the thiamine content of the egg. This is not strictly the case, although the variability of the data then presented did not warrant a discussion of it. It has long been known that there is a drop in the total weight of the egg as development proceeds. Tangl ('03) reported a loss of 17.9% although Murray's ('25) figure of 14.9% probably



represents a better average value. Our own series showed a weight loss of about 15% (8.1 to 16.5%). Unless the total concentration of thiamine at the end of development can be shown to be approximately 15% greater than that at the beginning of development, some loss of thiamine in the egg must have occurred. When cognizance is taken of the weight change the data show that a small loss has actually occurred. This is graphically illustrated by figure 1, and the loss appears to be approximately 20%.

This is a figure of interest because considerable respiratory activity can be measured during development (compare Romanoff, '41). In fact about one-third of the energy originally available in the egg has been used by the time of hatching. The relation of the energy used during development to that originally present in the egg, the apparent energetic efficiency (AEE), has been reported at 63 to 67 for the embryo chick (Tangl, '03; Murray, '26). If much of this energy exchange involved carbohydrate metabolism, as in non-embryonic forms, considerable thiamine would necessarily be required. However, the fact that the thiamine loss seems to be low in the developing chick embryo is consistent with the data of Needham ('31) showing fat to be the predominant energy source. The maintenance energy metabolism apparently involves a relatively small percentage of carbohydrate compared with the same respiratory exchange for the chick or adult. This is particularly true after the 10th day of incubation, when evidences of increased fat metabolism can be found (Murray, '26; Tallarico, '08; Riddle, '16). Before this time the total mass of the embryo is so small that its limited use of carbohydrate would not be reflected in our macro assays.

The results do not confirm the conclusion of Westenbrink and Van Leer ('41) that most of the thiamine originally present in the egg fails to appear in the embryo. In fact, most of the original thiamine is still present in the one-day-old chick which has had no opportunity to feed. The small loss which can be detected and attributed to developmental re-

quirements does not alter this general conclusion. It was considered possible that the eggs studied here contained so much more thiamine than the European eggs used by the above workers that the utilization of thiamine by the embryo was obscured. Study of their original paper when it became available showed that this was not the case, because all of the eggs used were similar to ours in thiamine content. Also, values obtained by us for incubated "low-thiamine eggs" refute this possibility.<sup>3</sup> The suggestion previously made that the discrepancy might lie in the extra-embryonic structures and fluids has not been borne out in the present studies. In the work presently reported, the thiamine content of the embryo doubled between the 16th and the 18th days and tripled between the 16th and 20th days. The actual content of the one-day chick was nearly double that of the 18-day embryo. It is apparent that most of the thiamine enters the embryo after the 16th day under the conditions of incubation in Rochester. If the 18-day embryo in Holland more nearly approximated the 16-day embryos of Rochester, the discrepancy in embryo thiamine content at this stage would not be great. We could then conclude that Westenbrink and Van Leer simply missed the great acquisition of thiamine by the embryo in the last few days of development. This would not, however, explain their report that the "eighteen day embryo plus yolk" contains only 35% of the amount initially present in the egg.

#### SUMMARY

The changing thiamine concentration of the embryo and the remainder of the egg contents has been determined by the macro fermentation method for embryonated eggs of White Leghorn fowls. The total thiamine concentration of

<sup>3</sup> Three hens were isolated and put on a diet containing less than  $0.6 \mu\text{g}$  of thiamine per gram. The thiamine concentration of the eggs laid by these hens was determined. The average thiamine values for eggs laid on successive days of the experiment were 118, 97, 74, 72, 54, and  $34 \mu\text{g}$  per 100 gm. Two eggs laid on the 7th day of the experiment were analyzed after 19 days of incubation and found to contain  $35 \mu\text{g}$  per 100 gm.

the egg contents does not change greatly during incubation. However, by correcting for the loss of weight of the egg during development, a drop of about 20% is found and attributed to the needs of developmental metabolism.

The concentration of thiamine in the embryo gradually increases in an exponential fashion. The concentration in the remainder of the egg rises a little after the 7th day and begins to drop rapidly after the 15th day. The thiamine content of the remains as represented by the spare yolk of the day-old chick was found to have dropped to about 20% of the original concentration in the egg and about 7% of the original concentration in the yolk.

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# THE INFLUENCE OF HIGH LEVELS OF FAT WITH SUBOPTIMUM LEVELS OF RIBOFLAVIN ON THE GROWTH OF CHICKS<sup>1</sup>

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THREE FIGURES

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The literature concerning the effect of fat in the diet on the riboflavin requirement is controversial. Potter, Axelrod and Elvehjem ('42) reported that lard had no effect on the riboflavin requirement of dogs fed sucrose diets. Mannering, Lipton and Elvehjem ('41) fed growing rats diets containing 25 to 40% "fat" and found that the riboflavin requirement increased with the fat level in the diet. In these experiments fat replaced dextrin. In a continuation of this work, Mannering, Orsini and Elvehjem ('44) concluded that the effect was due to the influence of the fat (lard and commercial hydrogenated cottonseed oil<sup>2</sup>) on decreasing the intestinal synthesis of riboflavin, since, when sucrose, which does not enhance intestinal synthesis, was substituted for dextrin the same reduced growth rate resulted. Tange ('41) has also reported an increased requirement of rats for riboflavin on high fat diets. Euler et al. ('42) have reported that the administration of linoleic acid to rats on a low riboflavin-low fat diet produced death in most animals.

<sup>1</sup> This work was carried on with the support of the Office of Naval Research under a contract with the Texas A. and M. Research Foundation.

<sup>2</sup> Crisco.

Since previous work on the requirements for riboflavin were with mammalia, it was of interest to extend studies of this nature to an avian species.

#### EXPERIMENTAL

The basal diet used had the following percentage composition: Wood pulp 3.0, cerelese 63.2, purified casein 18.0, gelatin 10.0, salt mixture 5.0, L-cystine 0.3 and fish liver oil 0.5. The vitamin supplement, in mg per 100 gm, was composed of thiamine hydrochloride 0.6, calcium pantothenate 2.5, choline chloride 250, nicotinic acid 7.0, pyridoxine hydrochloride 0.8, biotin 0.03, inositol 125, para-aminobenzoic acid 0.3, tocopherol 5.0, folic acid 0.25, 2-methyl-1,4-naphthoquinone 0.15. Fat was substituted for cerelese on a percentage basis. Not more than two weeks' feed was prepared at any one time, nor more than enough for three days' consumption placed in the feed troughs.

The chicks used were New Hampshire Red-White Leghorn crosses. In all experiments the chicks were distributed among the groups according to weights, so that there were the same number of light and heavy chicks in each group and the average weights in the groups were approximately the same.

In the first experiment (experiment I, table 1), two groups of 10 one-day old chicks were placed on the low fat and two groups on a 20% lard diet. One group on the low fat diet and one on the high fat diet were supplemented with 2  $\mu$ g of riboflavin per gram, and the other two groups received 4  $\mu$ g of riboflavin per gram of diet. Since the basal diet contained approximately 0.5  $\mu$ g per gram, the total riboflavin contents were thus 2.5 and 4.5  $\mu$ g per gram. These levels of riboflavin were selected because the 2.5  $\mu$ g level is inadequate while the 4.5  $\mu$ g one would normally meet the requirements for maximum growth.

The chicks were maintained on experiment for 40 days. At the end of that time the average gains of the chicks on the low riboflavin diets were 414 and 456 gm on the low and high fat diets, respectively. The average gains on the high ribo-

TABLE 1  
The effect of fat in the diet on the growth of chicks receiving various levels of riboflavin

EXPERIMENT	GROUP	DIETARY REGIMENS		NO. OF CHICKS	AGE OF CHICKS			LENGTH OF EXPERIMENT	GAIN	STANDARD DEVIATION
		Fat	Riboflavin		Beginning	Termination	days			
			$\mu\text{g}/\text{gm}$		days	days	days	$\text{gm}$		
I	1	None added	2.5	10	1	41	40	414	$\pm 14$	
	3	20% lard	2.5	10	1	41	40	456	$\pm 37$	
	2	None added	4.5	10	1	41	40	543	$\pm 21$	
	4	20% lard	4.5	9	1	41	40	531	$\pm 50$	
II	1	None added	0.5	12	14	33	19	65	$\pm 5.1$	
	2	20% CSO <sup>1</sup>	0.5	10	14	33	19	5.8	$\pm 2.7$	
	1	None added	4.5	9	35	47	12	228	$\pm 17$	
	2	20% CSO <sup>1</sup>	4.5	9	35	47	12	210	$\pm 15$	
III	1	None added	4.5	6	14	29	15	129	$\pm 13$	
	2	20% CSO <sup>1</sup>	4.5	6	14	29	15	121	$\pm 17$	
	3	None added	0.5	9	14	29	15	37	$\pm 2.7$	
	5	20% lard	0.5	10	14	29	15	30	$\pm 4.1$	
	6	20% Spry	0.5	10	14	29	15	26	$\pm 3.2$	
	4	20% CSO <sup>1</sup>	0.5	10	14	29	15	3.1	$\pm 2.1$	
IV	1	None added	4.5	9	14	27	13	162	$\pm 11$	
	2	5% CSO <sup>1</sup>	4.5	10	14	27	13	169	$\pm 11$	
	3	10% CSO <sup>1</sup>	4.5	10	14	27	13	174	$\pm 12$	
	4	20% CSO <sup>1</sup>	4.5	10	14	27	13	177	$\pm 16$	
	1	None added	0.5	9	27	39	12	140	$\pm 11$	
	2	5% CSO <sup>1</sup>	0.5	10	27	39	12	90	$\pm 10$	
	3	10% CSO <sup>1</sup>	0.5	10	27	39	12	64	$\pm 15$	
	4	20% CSO <sup>1</sup>	0.5	10	27	39	12	51	$\pm 8.1$	
V	1	None added	1.0	11	1	18	18	32	$\pm 1.6$	
	4	20% CSO <sup>1</sup>	1.0	9	1	18	18	15	$\pm 1.6$	
	2	None added	2.0	9	1	18	18	61	$\pm 5.6$	
	5	20% CSO <sup>1</sup>	2.0	10	1	18	18	22	$\pm 1.4$	
	3	None added	3.0	11	1	18	18	112	$\pm 5.9$	
	6	20% CSO <sup>1</sup>	3.0	10	1	18	18	50	$\pm 3.8$	

<sup>1</sup>Cottonseed oil. Wesson oil was used throughout.

flavin level were 543 and 531 gm on the low and high fat rations. The data analyzed statistically showed that there were no significant differences attributable to the lard.

An exploratory experiment (experiment II, table 1) was then performed, using a 20% cottonseed oil<sup>3</sup> diet instead of

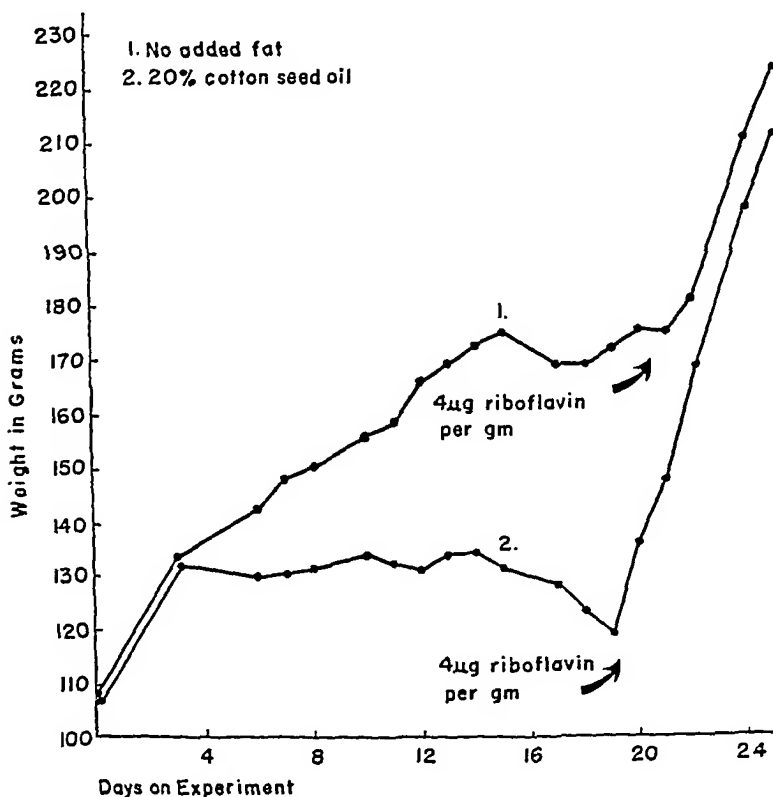


Fig. 1 Growth of 14-day-old chicks on low riboflavin diets containing 20% cottonseed oil and no added fat.

lard. The chicks had been on a stock diet for 14 days before being placed on the experimental ration. Only the fat content was varied, no riboflavin supplements being added for the first 19 days. Twelve chicks were started in each group. The results were strikingly different from those obtained on the

<sup>3</sup> Refined cottonseed oil available commercially as Wesson Oil.



experiment in which lard was fed. As may be noted in figure 1, the chicks on the cottonseed oil ration did not gain after the third day and began to lose weight sharply after the 14th day. The chicks on the low fat ration gained during the first 15 days and then failed to gain. Four micrograms of riboflavin per gram were then added to the cottonseed oil diet on the 19th day and to the low fat ration on the 21st day. The chicks subsequently gained rapidly, at about the same rate. As is seen in table 1, experiment II, on the 0.5  $\mu$ g riboflavin level the low fat group gained 65 gm in 19 days, as compared to 5.8 gm for the cottonseed oil group. After supplementation with riboflavin the gains were 228 and 210 gm, respectively, in 12 days. This experiment indicated clearly a deleterious effect of cottonseed oil in a low riboflavin ration, which was corrected by the inclusion of riboflavin.

An experiment was then designed to confirm the observed responses of chicks to low riboflavin diets high in lard and cottonseed oil, and to test a hydrogenated vegetable oil. The effects of low fat and high cottonseed oil diets containing adequate riboflavin were also studied. The chicks were placed on experiment after 13 days on a stock diet. The results are presented in figure 2 and table 1, experiment III.

From figure 2 it may be seen that the low fat and high cottonseed oil groups of 6 chicks each, which received a supplement of 4  $\mu$ g of riboflavin per gram (groups 1 and 2) grew at approximately the same rate. The low fat, lard and hydrogenated vegetable oil<sup>4</sup> groups of 10 chicks each which received no added riboflavin (groups 3, 5 and 6) gained slowly for 12 days and then began to lose weight. The cottonseed oil group of 10 chicks which received no added riboflavin (group 4) gained for only two days, maintained approximately the same weight for 10 days and then began to decline. On the 15th day 4  $\mu$ g of riboflavin per gram was added to the riboflavin-deficient diets. The resulting gains were as great in the cottonseed oil group as in the others.

<sup>4</sup>Spry.

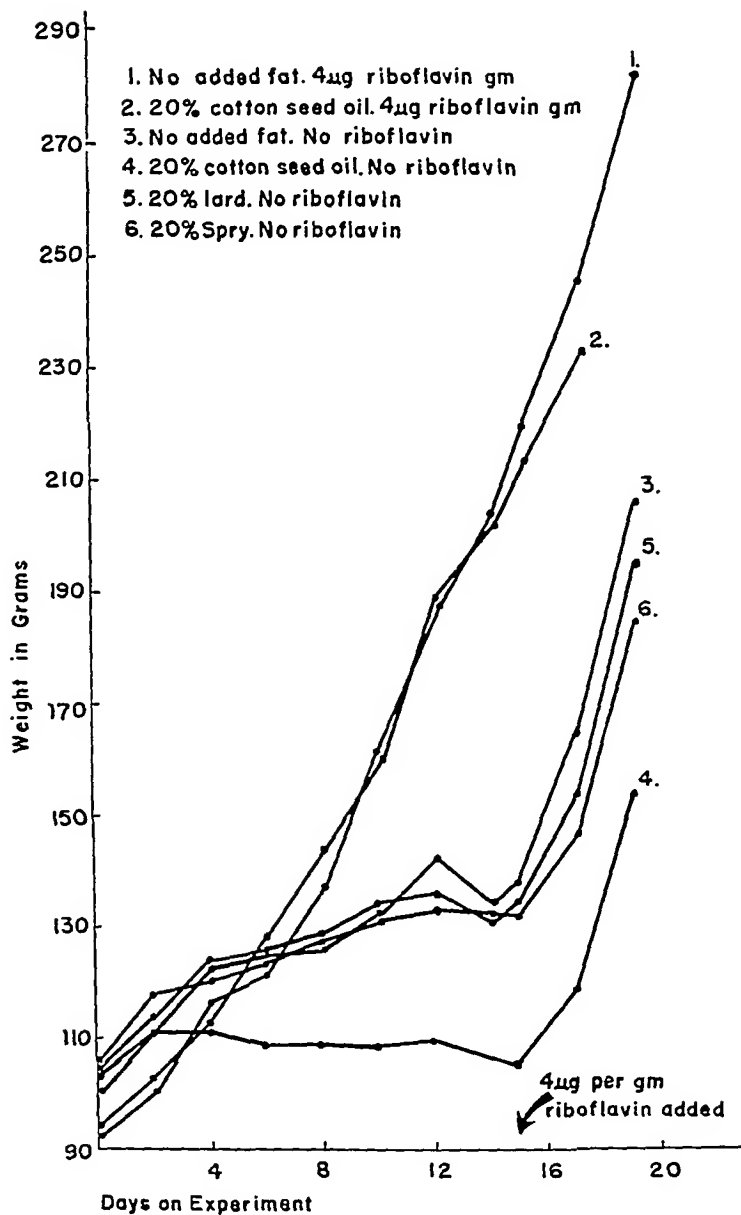


Fig. 2 Growth of 14-day old chicks on low and high fat and low and high riboflavin diets.

As shown in table 1, the average gain for the 15 days of depletion was approximately 30 gm by the chicks receiving lard, hydrogenated vegetable oil or no fat but was only 3 gm by those receiving cottonseed oil.

The deleterious effect of cottonseed oil which can be prevented or corrected by the inclusion of riboflavin is thus confirmed and contrasted to the comparatively innocuous effect of low fat, lard or hydrogenated vegetable oil rations.

To test the possibility that the development of rancidity might be responsible for the unfavorable effect of cottonseed oil, Nordihydroguaiaretic acid and citric acid were each added to this diet at a 0.05% level (experiment IV, table 1). Two-week-old chicks were used. Cottonseed oil was fed at levels of 5, 10 and 20%. Four micrograms of riboflavin per gram were mixed in the diets for the first 14 days and then discontinued. The average gains per chick on the 13th day were 162, 169, 174, and 177 gm on diets containing no fat and 5, 10 and 20% of cottonseed oil, respectively.

The riboflavin was then removed from the diets and on the 12th depletion day the respective average gains were 140, 90, 64 and 51 gm per chick on the low fat and 5, 10 and 20% cottonseed oil diets. Thus the protective role of riboflavin was again confirmed, and the deleterious effects of cottonseed oil shown not to be due to rancidity. Furthermore, the higher the level of cottonseed oil in the diet, the lower the rate of growth (fig. 3).

Since in all experiments with cottonseed oil the chicks had been placed on experiment after two weeks on a stock diet, it was decided to test the effects of cottonseed oil starting with day-old chicks. Three levels of riboflavin, 1.0, 2.0, and 3.0  $\mu$ g per gram of feed, were used with low fat and 20% cottonseed oil diets. The growth data are summarized in table 1, experiment V. The better growth of the low fat groups is obvious.

The chicks receiving diets containing 20% of cottonseed oil with all three levels of riboflavin made less efficient gains than the chicks on the diets with no added fat. The grams of feed required to produce 1 gm of gain on the 20% cotton-

seed oil diets were 3.37, 2.90 and 2.04, respectively, for the 1, 2 and 3  $\mu\text{g}$  levels of riboflavin. The feed consumed per gram of gain by chicks on the diets with no added fat for the corresponding riboflavin levels were 2.71, 2.21 and 1.36 gm. On the basis of the energy content of the diets, the high fat diets might have been expected to give the most efficient gains:

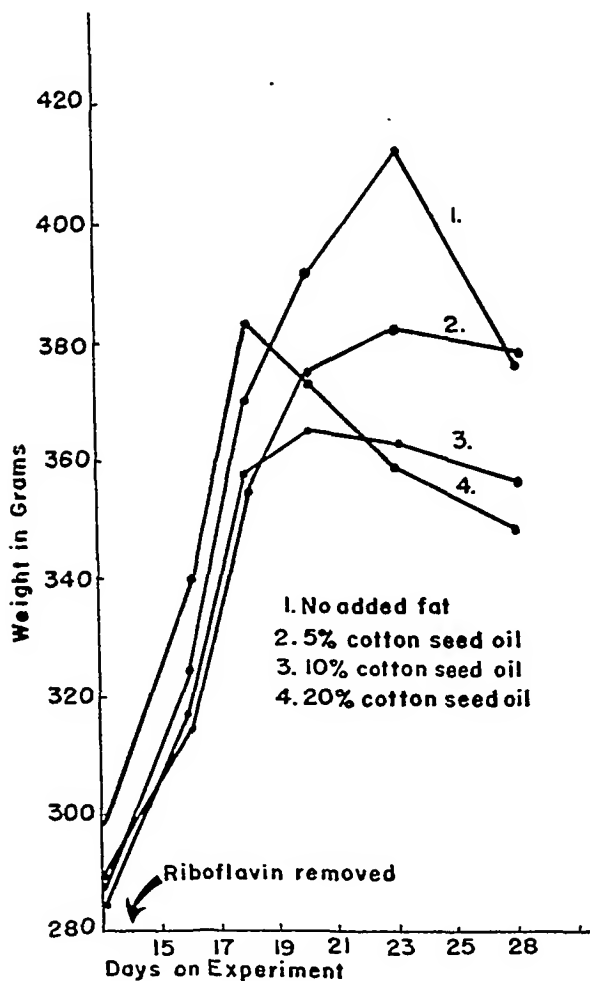


Fig. 3 The effect of graded levels of cottonseed oil on the growth of 14-day-old chicks upon removing riboflavin from the diets.

On the 18th day of the experiment the chicks were sacrificed and the livers of those receiving 1 and 3  $\mu\text{g}$  of riboflavin per gram of diet assayed for riboflavin by the method of Roberts and Snell ('46). The riboflavin content of the livers was not affected by the addition of fat to the diets. On the 1  $\mu\text{g}$  level of riboflavin, the average riboflavin content of the livers on the low fat diet was 7.2  $\mu\text{g}$  per gram of fresh liver, as compared with 7.8  $\mu\text{g}$  for the chicks on the diet containing 20% of cottonseed oil. On the diets containing 3  $\mu\text{g}$  of riboflavin, the average levels in the liver were 15.9 on the low-fat diet and 13.7 on the diet containing cottonseed oil. The lower riboflavin content of the liver on the low levels of intake is in accord with the finding of Clandinin ('46).

The pronounced inhibitory effect on growth and the inefficient use of feed resulting from the inclusion of 20% cottonseed oil in a diet containing a suboptimum amount of riboflavin are again clearly demonstrated.

#### DISCUSSION AND SUMMARY

Chicks receiving moderately high levels of refined cottonseed oil in diets low in riboflavin cease growing much sooner than those on a similar diet that contains no added fat. When 4  $\mu\text{g}$  of riboflavin per gram of feed are added to the diets, the growth response is essentially the same irrespective of whether the diets contain fat. Lard did not retard the rate of growth, nor did a commercial hydrogenated vegetable fat.<sup>3</sup> The response to cottonseed oil does not appear to be due to the development of rancidity since means were taken to prevent its formation, including the addition of anti-oxidants and refrigeration of the feed.

The chemical difference between cottonseed oil on the one hand and lard and hydrogenated vegetable oil on the other is the high concentration of linoleic acid in the former. Since there is no known reason for believing that the unsaturated acids require more riboflavin for their metabolism than the

<sup>3</sup> See footnote 4, page 251.

saturated it would seem more probable that the unsaturated acids interfere more with the intestinal synthesis of riboflavin.

The alternative possibility, however, must still be considered. Lehninger has recently shown ('48) that cytochrome is an essential constituent of the enzymatic system for the oxidative catabolism of fatty acids. Since riboflavin is a constituent of the enzyme system which catalyzes the reduction of the oxidized form of cytochrome C (Haas, Horecker and Hogness, '40), it becomes apparent that this vitamin has a role in fatty acid catabolism and an increased requirement might be expected on a high fat diet.

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# EFFECT OF FAT IN THE DIET OF RATS ON THEIR GROWTH AND THEIR EXCRETION OF AMINO ACIDS<sup>1</sup>

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The importance of the interrelationships among the various nutrients of the diet has been increasingly realized during the last decade. Among these relationships, dietary fat has been found to influence energy and protein metabolism and various physiological functions. Increasing the fat content of isocaloric diets from 2 to 30% resulted in an increase in the rate of weight gain and a decrease in the heat production of rats (Forbes, Swift, Elliott and James, '46; Forbes, Swift, James, Bratzler and Black, '46). Rats fed diets low in protein made more rapid and efficient gains when 30% of fat was included in the diet than when only 2% was included (French, Black and Swift, '48). Deuel et al. ('47)<sup>1</sup> have shown that feeding rats diets containing from 5 to 40% of fat results in better growth, greater physical capacity, and better reproductive and lactation performance than does the feeding of a low fat diet supplemented with methyl linoleate. The low fat diets used in previous investigations reviewed by the present authors were provided with the essential unsaturated fatty acids, so the beneficial effects of the fats must be attributed to other factors. Annegers and Ivy ('47) have shown that increasing the fat content of the diet delays gastric

<sup>1</sup> This work was carried on with the support of the Office of Naval Research under a contract with the Texas A. & M. Research Foundation.

evacuation. This might favor more complete digestion of the various nutrients.

The experiments reported here were designed to examine the effect of fat in the diet on the utilization of the protein component of the diet under conditions of isocaloric feeding and ad libitum feeding. The criteria used were rate of gain, gain per gram of protein consumed, and the fecal and renal excretion of phenylalanine, valine, lysine and methionine. The rationale of the latter was the finding that the amounts of amino acids excreted in the urine are influenced by the quality of the protein ingested (Sauberlich, Pearce and Baumann, '48). With proteins of high biological value the urinary excretion of amino acids was much lower than when proteins of a low biological value were fed. These findings indicate that one means of measuring the efficiency of utilization of proteins is by the renal excretion of amino acids. The size of the three-day sample of urine and feces did not permit assays for all of the essential amino acids.

#### EXPERIMENTAL

The percentage composition of the fat-free (low fat) diet was: purified casein 20, sucrose 76 and salts mixture 4. The fats used in these experiments were lard and a refined corn oil. The components of the diets containing fat were purified casein 22%, sucrose 66%, salts mixture 4% and either corn oil or lard 8%. One gram of the low fat diet was calculated on the basis of physiological heat values for protein, fat and carbohydrate to provide energy and protein equivalent to 0.906 gm of the diets containing fat. Vitamins were added to the diets in mg per 100 gm of diet as follows: tocopherol acetate 0.5, vitamin D<sub>3</sub> 0.2, vitamin A 0.2, 2-methyl-4-naphthoquinone 0.1, thiamine 0.25, riboflavin 0.3, pyridoxine 0.25, Capantothenate 2, choline chloride 100, niacin 0.5, inositol 100, pteroylglutamic acid 0.2 and biotin 0.01. The diets were stored in a refrigerator. Each rat was given approximately 30 mg of ethyl linoleate daily by means of a medicine dropper. This is adequate to meet the needs of the rat for the essential



polyunsaturated fatty acids. The ethyl linoleate was given to the rats receiving the diets containing fat to insure as much uniformity as possible in the treatment of the animals.

White rats of the Sprague-Dawley strain were used. To avoid variations due to sex differences, only male rats were used. The details of the method of feeding will be given for each series as the results are discussed.

Quantitative collections of the urine and feces were made for three consecutive days. The period of collection of feces and urine was between the beginning of the third week and end of the 4th week, unless otherwise indicated. It was necessary to stagger the collections over the two-week period. An equal number of rats from each group were always in the metabolism cages at the same time. The urines were filtered and stored in a refrigerator. The feces were dried and ground for analyses.

Microbiological assays for the amino acids in the feces and urine were made on the hydrolyzed samples and represent, therefore, the total content of phenylalanine, valine, lysine and methionine. Methionine and lysine were determined with the test organism *Leuconostoc mesenteroides* P-60, using the procedure of Lyman et al. ('46). Valine and phenylalanine were assayed with *L. arabinosus* (Schweigert et al., '44) with the modification that 0.02  $\mu$ g folic acid and 1  $\mu$ g pyridoxamine were added per 10 ml of medium.

#### *Ad libitum feeding*

In this series the rats were fed the three diets ad libitum in individual cages, thereby permitting individual feed consumption records. There were 12 male rats in each group with an initial weight of approximately 40 gm. The gains for the 4-week period and gain per gram of protein consumed, together with the standard deviations, are shown in table 1. The rats fed the diet low in fat made an average gain of 76 gm, as compared with 107.8 gm for the group receiving corn oil in the diet and 105.2 gm for the group with lard in the

diet. The difference in the gains made by the group fed the low fat diet and the groups fed the diets containing 8% of fat was highly significant statistically. While the rats receiving 8% of fat in the diets made more gain per gram of protein consumed than the group fed the low fat diet, these differences are not statistically significant. The corn oil and lard groups consumed more feed than the low fat group and the lower food consumption is without doubt a factor in the less rapid growth of the latter group. The superior growth performance of the rats fed diets containing fat is in accord with previous observations (Deuel et al., '47; Scheer et al., '47).

TABLE 1

*Growth of rats on diets containing corn oil, lard and no fat*

DIETARY REGIMEN	GAIN IN 4 WEEKS		GAIN PER GRAM PROTEIN CONSUMED	
	Mean	S.D. <sup>1</sup>	Mean	S.D. <sup>1</sup>
	<i>gm</i>		<i>gm</i>	
No fat	76.0	17.3	1.4	0.3
Corn oil	107.8	15.1	1.6	0.3
Lard	105.2	15.8	1.7	0.3

<sup>1</sup> Standard deviation.

### *Excretion of amino acids*

This series was designed to measure the effect of fat in the diet on the renal and fecal excretions of phenylalanine, valine, lysine and methionine. Data were also obtained on growth and efficiency of utilization of feed. There were 15 male rats in each group with an initial average weight of approximately 42 gm. Each animal was fed in an individual cage and an accurate record was kept of the amount of food consumed. The allowance of food was such that each animal in all groups received essentially the same amount of protein and energy. That this was essentially achieved among the three groups is evidenced by the fact that the average amounts of protein consumed per rat for the 4-week period showed a maximum difference of only 0.2 gm. The urine and feces

were collected separately over a three-day period. The number of metabolism cages available and the time sequence did not permit obtaining excretion data on all of the 15 animals in each group. Collections were made on 12 rats in each of the three groups. In a few cases the size of the sample of feces or urine was not sufficient to permit assays for all of the 4

TABLE 2

*Effect of fat in the diet of rats on their growth and their excretion of amino acids*

CATEGORY OF INTEREST	DIETARY REGIMEN					
	No fat		Corn oil		Lard	
	Mean	S.D. <sup>1</sup>	Mean	S.D. <sup>1</sup>	Mean	S.D. <sup>1</sup>
Gain/4 weeks (gm)	57.4	5.7	61.7	6.1	60.2	6.1
Gain/gm protein consumed (gm)	1.7	0.1	1.8	0.1	1.8	0.1
Phenylalanine excreted/3 days in:						
urine (mg)	2.0	1.4	1.3	0.6	1.3	0.8
feces (mg)	4.9	1.7	4.1	2.3	4.3	1.6
Valine excreted/3 days in:						
urine (mg)	1.7	1.3	0.9	0.03	1.5	0.2
feces (mg)	10.2	3.0	7.0	1.4	8.7	2.1
Lysine excreted/3 days in:						
urine (mg)	2.4	1.2	1.8	0.6	2.0	0.8
feces (mg)	8.6	3.3	6.4	1.9	7.5	1.7
Methionine excreted/3 days in:						
urine (mg)	0.64	0.4	0.4	0.1	0.5	0.3
feces (mg)	3.0	0.9	2.1	0.6	2.5	0.9

<sup>1</sup> Standard deviation.

amino acids. The growth data in table 2 and the gain per gram of protein consumed are for 15 animals in each group, while the figures for amino acid excretions represent either 11 or 12 animals in each group.

The mean gain in weight of the rats fed the low fat diet for the 4-week period was 57.4 gm, as compared to 61.7 and 60.2 gm for the groups receiving corn oil and lard, respectively. These differences are not significant statistically.

The amounts of amino acids excreted in the feces were from two to 5 times greater than were excreted by the renal pathway. We have no data to indicate the proportion of the fecal amino acids that comes from ingested protein. It is probable that a substantial proportion originates from other sources. On the basis of work with humans Sheffner, Kirsner and Palmer ('48) have estimated that fecal amino acids represent to a large extent amino acids secreted into the lumen of the gastrointestinal tract as components of digestive enzymes and excreted as such or converted into bacterial protein before elimination from the body. Nevertheless, it may be assumed where significant differences occur that they are not artifacts, since amino acids of endogenous origin would not be expected to differ essentially with the various dietary regimens.

The fecal excretions of each of the 4 amino acids were less by the rats receiving corn oil in their diet than by the rats receiving the diet with no added fat. Similarly, the amounts of each of the 4 amino acids excreted in the feces were less on the diet containing lard than on the basal diet containing no fat. There was a tendency for the fecal excretion of the amino acids to be lower on the corn oil diet than on the lard diet, but most of these differences are of small magnitude and it is doubtful if much significance can be attached to them. Statistically the difference between the fecal excretion for the low fat diet and the corn oil diet was significant for lysine, highly significant for valine and methionine, and not significant for phenylalanine. One interpretation of this is that corn oil at a level of 8% in the diet favors the digestion and absorption of lysine, methionine and valine.

The average renal excretion by the rats receiving corn oil was 0.9 mg of valine and 0.4 mg of methionine for the three-day period. The corresponding figures for the rats not receiving fat in their diet are 1.7 and 0.6 mg, respectively, for valine and methionine. Differences in the urinary excretion of these two amino acids are significant statistically, while for phenylalanine and lysine the differences are not statisti-

cally significant. These results show that 8% of corn oil in the diet of rats enhances the efficiency with which methionine and phenylalanine are utilized by this animal.

### *Tissue replenishment*

This series was designed to measure the rate of tissue replenishment in rats fed a diet with no added fat and a diet containing 8% of corn oil. Rats that had been fed a stock

TABLE 3

*Effect of fat on the rate of weight gain and the excretion of amino acids during tissue replenishment*

CATEGORY OF INTEREST	DIETARY REGIMEN			
	No fat		8% corn oil	
	Mean	S.D. <sup>1</sup>	Mean	S.D. <sup>1</sup>
Gain/3 weeks (gm)	76.1	7.6	78.2	8.5
Phenylalanine excreted/3 days in:				
urine (mg)	5.5	2.4	5.3	3.7
feces (mg)	7.5	2.4	7.7	2.8
Valine excreted/3 days in:				
urine (mg)	8.1	4.5	6.6	3.3
feces (mg)	26.9	10.7	28.5	16.1
Lysine excreted/3 days in:				
urine (mg)	8.5	3.1	7.5	2.3
feces (mg)	11.9	3.0	10.9	4.0
Methionine excreted/3 days in:				
urine (mg)	2.8	1.36	2.3	1.2
feces (mg)	6.0	1.85	5.6	2.3

<sup>1</sup> Standard deviation.

ration until they weighed approximately 215 gm were then placed on a diet consisting of sucrose, salts, and vitamins at twice the levels used in the diets containing protein. During the depletion period each rat received 30 mg of ethyl linoleate daily. After they had lost between 30 and 40% of their weight they were divided into two groups of 10 rats each and placed in individual cages. The rats receiving the diet containing 8% of corn oil and those fed the diet containing no added

fat were fed at levels to provide equal protein and calorie intakes. They were kept on the experimental diets for three weeks, at which time their rate of gain reached a plateau. Between the 10th and 21st day of the experiment the urine and feces were collected individually for a three-day period.

The data on the rate of gain and the excretion of 4 amino acids are shown in table 3. The differences between the two groups with respect to rates of gain and the excretions of phenylalanine, valine, lysine and methionine were not significant statistically. It should be noted, however, that there is a tendency to excrete less amino acids when corn oil is included in the diet than when no fat is added to the diet. There was less lysine and methionine excreted in the feces by the rats receiving corn oil in their diet than by the rats receiving the diet with no added fat. The average renal excretion of each of the 4 amino acids was less by the group receiving corn oil than by the group with no added fat in the diet. The results using the replenishment technique are in the main in accord with the observations with young rats.

#### SUMMARY

Rats fed *ad libitum* a diet with no added fat and supplemented with ethyl linoleate gained 29% less than rats fed diets containing 8% of corn oil or lard. There was no significant difference in the gains of rats when the intakes of protein and energy were equalized.

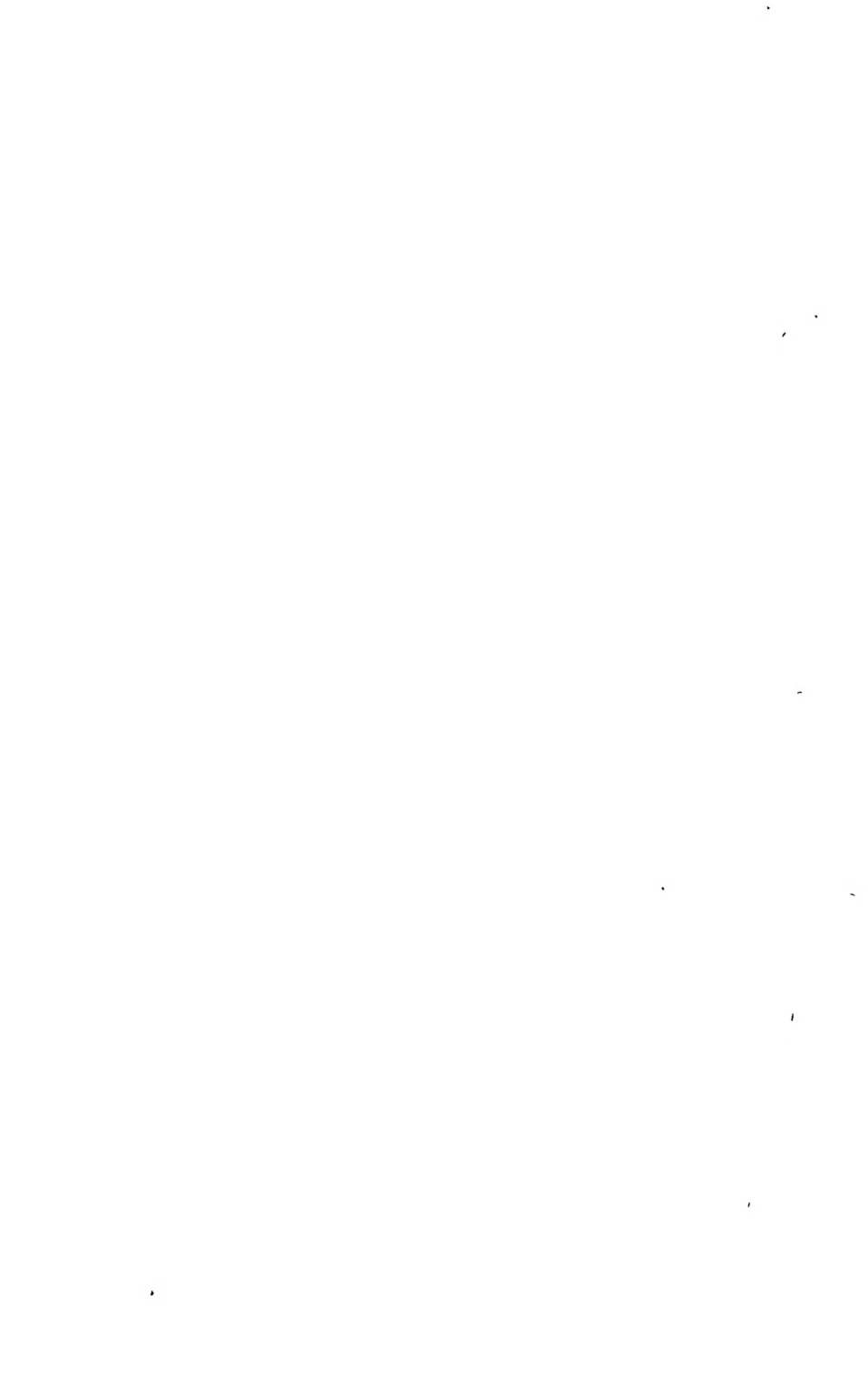
The fecal excretion of phenylalanine, valine, lysine and methionine was from two to 5 times greater than the excretion by the renal pathway. The fecal excretions of the 4 amino acids were less by the rats receiving corn oil in their diet than by rats receiving a diet with no added fat. The differences were statistically significant for valine, lysine and methionine. The urinary excretion of valine and methionine was significantly less by the rats fed the diet containing corn oil than by the rats fed a diet containing no added fat.

Mature rats that had been fed a depletion diet until they had lost approximately one-third of their weight were fed on

a regimen providing equalized intakes of protein and energy. One group was fed a diet containing 8% of fat and the other group a diet with no added fat. The results, on the basis of growth and excretion of amino acids, favored the diet containing corn oil. The differences, however, were small and not statistically significant.

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# THE DISTRIBUTION OF THIAMINE IN THE EMBRYONATED HEN EGG

## II. THE CONTENT OF EMBRYONIC TISSUES <sup>1</sup>

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ONE FIGURE

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Despite the great amount of embryological and biochemical work done on the chick embryo, studies of the vitamin content of the embryonic tissues are rare. Barnett and Bourne ('41) have studied the distribution of ascorbic acid in the embryo during the first 4 days of incubation. The concentrations of 8 of the vitamins of the B complex, including thiamine, have been determined by Williams et al. ('41) for the liver, brain and hearts of 12-day embryos and day-old chicks. Attempts to modify these values by changing the diet of the hen or injecting vitamins into the egg have been reported from the same laboratory (Taylor et al., '41).

Studies of the thiamine content of the entire egg and intact embryo have been discussed in a preceding paper (Scrimshaw, Porter and Scrimshaw, '49). In this report the thiamine concentration and content of the embryo were shown to increase in exponential fashion. The thiamine concentration of the egg does not change until it drops rapidly during the final two or three days of incubation. The total thiamine content of the egg appears to drop about 20% by the time of

<sup>1</sup> Aided by a grant from Swift and Co., Chicago, Illinois.

hatching. The yolk contains all of the thiamine present in the egg before incubation (compare Chick and Roscoe, '29; Ellis et al., '33; Bethke et al., '36). The concentration of the yolk thiamine falls from approximately 300  $\mu\text{g}$  per 100 gm at this time (Scrimshaw and Stewart, '44) to 23  $\mu\text{g}$  per 100 gm for the "spare yolk" of the day-old chick.

In the present study the distribution of thiamine in the 18-day chick has been studied by assay of brain, liver, muscle, heart, gastrointestinal tract, kidney, yolk, membranes and remainder. The vitamin content of certain of these organs has also been studied in the 15-day embryo and the day-old chick. These values are correlated with those previously reported for the embryo and the whole embryonated egg.

#### MATERIALS AND METHODS

The eggs<sup>2</sup> were incubated as described previously (Scrimshaw, Porter and Scrimshaw, '49). Thirty eggs of the desired age were usually selected and the embryo, yolk and remains carefully separated and weighed. The embryos were then dissected. Each kind of organ or tissue was then weighed and prepared as a single sample. Most of the samples were mixed immediately in a Waring Blendor, acidified to pH 4.00 or below with HCl and diluted to volume for storage at 5° C. Striated muscle samples were placed in a vacuum desiccator in the cold room and ground up in a mortar when dry. The powdered samples were then treated as above.

The assays were done by the modified macro fermentation method (Scrimshaw and Stewart, '44). Often the quantity of sample was too small to permit duplicate runs. Where a value has not been checked by either a duplicate assay or by the assay of the same organ in a different series, this is indicated in table 2. Each of the 18-day values is the average of several series. Only one series of 15-day embryos and one-day-old chicks were studied.

<sup>2</sup>The eggs and day-old chicks were supplied from White Leghorn hens by Roger's Farms, a nearby commercial poultry farm and hatchery.

## RESULTS

The distribution of thiamine in the embryonated 18-day egg is described first because it received the most complete study and can be used as a basis for comparison. In table 1 the mean thiamine concentration and range of values obtained for each of the egg components tested are given, together with the mean weight of each in an individual egg. It will be noted that the concentration in the liver is approximately 4 times

TABLE 1

*The concentration of thiamine in the tissues and organs of 15-day embryos and day-old chicks*

STRUCTURE	NO. OF SAMPLES ASSAYED <sup>1</sup>	MEAN WT. PER EGG	CONCENTRATION <sup>2</sup>	
		gm	$\mu\text{g/gm}$	
Brain	3	0.78	1.50	(1.42-1.58)
Liver	3	0.45	3.68	(3.40-4.05)
G.I. tract	2	1.66	0.80	(0.35-1.25)
Kidney	2	0.14	1.95	(1.78-2.12)
Heart muscle	4	0.17	0.82	(0.56-1.34)
Leg muscle	2	...	1.35	(1.13-1.57)
Yolk	3	15.0	1.12	(1.05-1.19)
Membranes and fluid	2	12.6	0.92	(0.23-1.60)
Total less embryo	1	29.2	0.91	
Total embryo	2	18.6	0.91	

<sup>1</sup> These are the number of separate samples made up of 20 to 30 embryos assayed in duplicate.

<sup>2</sup> Values are not corrected for variations in initial thiamine concentration because such adjustment would not greatly affect the gross range of values.

that in the whole embryo. Kidney and brain and skeletal muscle are also seen to be higher in thiamine concentration than the mixed whole embryo, while the heart is about the same. It is of interest that at 18 days of incubation the concentration of thiamine in the embryo, in the yolk, and in the remaining fluid and membranes is approximately the same.

When these data are compared with those for the embryo at 15 days and the newly hatched chick presented in table 2, certain characteristic differences and similarities are noted.

There appears to be no significant change in the heart thiamine concentration. The concentration in the brain seems to drop steadily with development. By the time of hatching, a definite drop in the thiamine of the liver, muscle, gastrointestinal tract and yolk has occurred. In general, the values for 15 days do not differ markedly from those for 18 days except for a previously noted increase in concentration of thiamine in the whole embryo with age.

TABLE 2

*The concentration of thiamine in the tissues and organs of 15-day embryos and day-old chicks*

STRUCTURE	15-DAY EMBRYO		1-DAY CHICK	
	Mean weight	Concentration	Mean weight	Concentration
	gm	$\mu\text{g/gm}$	gm	$\mu\text{g/gm}$
Brain	0.587	2.50 <sup>1</sup>	0.895	1.18 <sup>1</sup>
Liver	0.290	3.34	1.23	2.58
G.I. tract			4.7	0.63
Heart	0.127	0.87 <sup>1</sup>	0.295	0.96 <sup>1</sup>
Leg muscle				0.84
Whole embryo	13.7	0.75	36.2	1.09
Yolk	32.3	0.97 <sup>2</sup>	2.0	0.23

<sup>1</sup> Only a single assay of a 30-embryo sample was done because of the limited quantity of material. Other values represent duplicate assays of a single sample. Values are not corrected for variations in initial thiamine concentration.

<sup>2</sup> Includes entire non-embryo portion of egg contents.

The average initial concentration of thiamine varied from one series to the next, as did the mean initial weight of the egg contents.<sup>3</sup> The cumulative effect of these differences would so distort the graphical presentation of the organ thiamine contents that these values are corrected for this variation. One hundred micrograms of thiamine per 100 gm of whole egg and an average weight of 50 gm per egg were values close enough to the true averages to serve as convenient arbitrary standards. If the initial content of a series averaged 90  $\mu\text{g}$  of thiamine per 100 gm, individual tissue

<sup>3</sup> The average initial values for each series are given in the preceding paper.

values of this series were multiplied by 100/90. Similarly, an average initial egg content weight of 45 gm would result in all mean organ weights for the series being multiplied by 50/45. The adjusted results are plotted in figure 1. It will be seen that in general the content increases with development due to the increase in mass of the organ or tissue studied. Changes in concentration with development are not great enough to obscure this trend. This is particularly noticeable in the case of the liver and the gastrointestinal

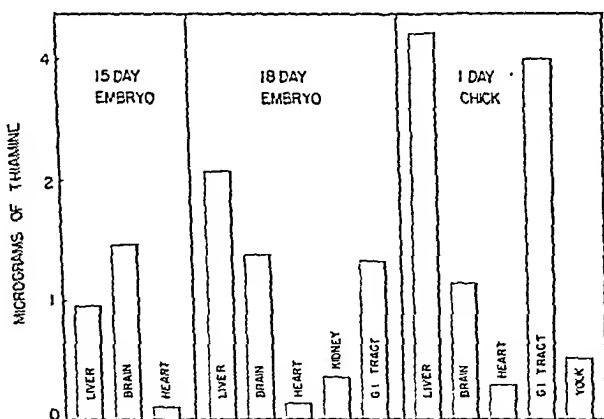


Fig. 1 Thiamine content of certain organs of the 15-day embryo, 18-day embryo and day-old chick. These values, corrected for initial variation in weight and thiamine concentration of the various series (see text), give the total thiamine to be found in the "average" organ at the stage indicated.

tract, which dropped considerably in concentration in the newly hatched chick but which later contained relatively large amounts of thiamine. Only in the case of the brain and, of course, the yolk, is a drop in thiamine content with development noted. If it had been possible to estimate the total mass of skeletal muscle, its thiamine content would undoubtedly have been the highest of any single tissue.

#### DISCUSSION

The single value reported by Williams, Taylor and Chel-delin ('41) for the liver of the 12-day embryo is two to three

times the one obtained by us for the 15-day embryo. However, their values are expressed in terms of dry weight and can be only approximately compared. Their figure for the liver of the day-old chick is in good agreement with ours. A single figure reported by Williams, McMahan and Eakin ('41) in a concurrent study was 1.9  $\mu\text{g}$  per 100 gm of fresh "chick" liver.

The concentration of thiamine in both the heart and brain of the 12-day chick embryo as reported by Williams et al. ('41) is greater than that found by us in the 15-day embryo. However, their results and ours seem reasonably consistent except for a much higher value cited by them for the 12-day embryo liver. The discrepancy may be due to a higher thiamine content of the younger liver, but it can also be accounted for by the variation to be expected in their single sample.

The concentration of thiamine in the embryo, weaned, and mature rat reported by the above workers in the same study shows marked qualitative and quantitative differences from the values found for the hen.

In some cases the functional significance of the distribution of thiamine described above will be apparent. A high concentration in the liver is consistent with the metabolic importance of this organ. Similarly, nervous tissue and kidney might be expected to show a high thiamine concentration. The higher thiamine value for the 18-day chick muscle as compared with that of the young chick is not so easily understood. Perhaps the considerable muscular activity of the young chicks during and after hatching had depleted the thiamine stored before this time.

The steady drop in the thiamine content of the yolk is of interest since by the time of hatching the total thiamine remaining is only one-sixth of that in the gastrointestinal tract to which the yolk is attached. In fact, it has only slightly more thiamine than the much smaller heart. Although originally containing all of the thiamine in the egg at a concentration 4 times that of the final embryo, the yolk has lost all significance as a thiamine store by the time of hatching.

The changes that take place in the embryo during the last three days of development are great. At 18 days the embryo still makes up only about half of the total weight of the egg contents and a corresponding proportion of the thiamine. However, the newly hatched chick has incorporated much of this 50% of both thiamine and tissue. These changes which take place during the final days of incubation are difficult to study because of the variation among individual embryos and technical difficulties in handling samples with much foam and feathers. Considerable additional work is required on these critical final days.

Although certain trends can be noted in the above data with respect to the thiamine content and concentration of embryonic organs and tissues, detailed analysis must await further study. Indeed, only with increased knowledge of the role of thiamine in development and metabolism can these results be satisfactorily interpreted.

#### SUMMARY

The thiamine concentration and content of the organs and tissues of 15-day and 18-day chick embryos, and of day-old chicks, were determined by the macro fermentation method. In the 18-day embryo the liver showed the highest concentration (4.5  $\mu\text{g}/100\text{ gm}$ ), followed by kidney (2.4), brain (1.8), muscle (1.8), gastrointestinal tract (1.0), and heart (1.0). The embryo at this stage showed a concentration of 1.1, the yolk 1.5, with the membranes and fluids intermediate. The corresponding values for the 15-day embryo are similar but those for the one-day chick are generally lower.

The total content is greater in the brain at 15 days than in the liver (1.5 as compared to 1.0  $\mu\text{g}$ ). By 18 days the liver contains the most thiamine (2.0  $\mu\text{g}$ ), followed by brain (1.4), gastrointestinal tract (1.3), kidney (0.3), and heart (0.1). In the day-old chick, liver and gastrointestinal tract have high thiamine contents (3.2 and 2.9), followed by brain (1.1), yolk (0.5) and heart (0.3). These values are compared with those previously reported for embryo and adult tissues.

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# CARIOUS LESIONS IN COTTON RAT MOLARS

## III. EFFECT OF THE PARTICLE SIZE AND THE CONSISTENCY OF THE PURIFIED RATION ON THE INCIDENCE AND TYPE OF CARIOUS LESIONS

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SIX FIGURES

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When cotton rats (*Sigmondon hispidus hispidus*) were maintained on a purified diet for 14 weeks after weaning, their molar teeth were found to have been susceptible to the initiation and development of carious lesions (Shaw, Schweigert, McIntire, Elvehjem and Phillips, '44). When the sucrose in this ration was replaced by dextrin or starch, the amount of tooth decay in cotton rats maintained on the resulting rations was much lower than in litter mates fed the sucrose ration (Schweigert, Shaw, Phillips and Elvehjem, '45). The feeding of mineralized whole milk as the sole source of nutrients, or of diets produced by the isocaloric replacement of part of the sucrose by lard, butterfat or casein, resulted in a decreased initiation and development of carious lesions (Schweigert, Shaw, Zepplin and Elvehjem, '46).

The effect of mastication on dental caries in the cotton rat was studied by extraction of the molars in one upper quadrant prior to the beginning of the experiment (Shaw, '47). The results indicated that there was a much less important effect of mastication of the purified diet upon the

dental caries attack rate than had been observed previously in the white rat fed the coarse, Hoppert-Webber-Canniff diet (Sognnaes, '41; Ginn, '42). However, cotton rats fed either the purified diet or a ration composed of whole milk solids in liquid form had substantially lower average numbers and average extents of carious lesions than litter mates fed the same rations in dry form (Anderson, Smith, Elvehjem and Phillips, '48).

Mechanical lesions are not known to precede the initiation of tooth decay in human teeth. Therefore, for any animal to be used for fundamental caries research it is of considerable importance to explore the relationships of diets with a wide variety of physical properties to the maintenance of teeth. The present series of experiments was conducted with cotton rats to study: (1) the morphology of the carious lesions in relation to tooth structure and masticatory stresses; and (2) the effects of diets of varying particle size and varying consistency on the production of tooth decay.

#### EXPERIMENTAL

The cotton rats used in these experiments were the offspring of animals which had been selected for breeding purposes through several generations because of their high and predictable susceptibility to tooth decay. Stock colony cotton rats were fed the ration composed of natural foodstuffs described previously (Shaw, '47). From weaning until the termination of an experiment, control and experimental rats were housed in individual cages with floors made of coarse wire screen.

Ration 100, a slight modification of the caries-producing diet of Shaw, Schweigert, McIntire, Elvehjem and Phillips ('44), was used as the basal ration. The composition of ration 100 and its various isocaloric modifications are presented in table 1. When these rations were offered ad libitum, with ample drinking water, a high percentage of the rats grew well and seemed to be normal in activity and appearance for the duration of the experimental periods. Some

TABLE 1  
Composition of ration in grams of each constituent

CONSTITUENTS	R A T I O N															
	100	120	130	140	104	134	144	100A	100B	120B	130A	130B	140A	101	102	
Sucrose	67	58	49	40				67	67	58	49	49	40	..	..	
Powdered sugar <sup>1</sup>					67	49	40							..	..	
Dextrin — fine														67	..	
Dextrin — coarse															67	
Casein <sup>2</sup>	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	
Corn oil <sup>3</sup>	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	
Corn oil <sup>4</sup>																
Lard	4	4	8	12		8	12	4	4	4	4	4	4	4	4	
Salt mixture <sup>4</sup>	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	
1:20 liver concentrate	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	
Whole liver substance	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	
Cellu flour								10	20	20	10	20	10	.	..	
Fat content as per cent by weight of digestible components	4.8	9.1	13.8	19.1	4.8	13.8	19.1	4.8	4.8	9.1	13.8	13.8	19.1	4.8	4.8	
Carbohydrate content as per cent by weight of digestible components	64.4	58.6	52.1	44.9	64.4	52.1	44.9	64.4	64.4	58.6	52.1	52.1	44.9	64.4	64.4	

<sup>1</sup> Three per cent corn starch added to prevent caking.

<sup>2</sup> "De-vitaminized," casein, Sheffield Farms, New York. Each 24 gm had had incorporated in them 350 µg of thiamine hydrochloride, 350 µg of pyridoxine hydrochloride, 2.0 mg of calcium pantothenate, 2.5 mg of nicotinic acid, 100 mg of choline chloride, 100 mg of inositol, and 30 mg of para-aminobenzoic acid.

<sup>3</sup> Mazola — Each 5 gm had had incorporated into them 1.1 mg of beta-carotene, 300 I.U. of irradiated ergosterol, 0.6 mg of 2-methyl-1,4-naphthoquinone, and 5.0 mg of alpha-tocopherol.

<sup>4</sup> J. Dent. Res., 26: 47, 1947.

deaths occurred, due usually to intercurrent infections, occasionally to the formation of hair balls in the stomach, but independent of the composition of the ration.

In the first experiment cotton rats were maintained on ration 100 for varying periods of from 6 to 14 weeks to provide histologic material. Litter mates were sacrificed at successive intervals by ether anesthesia, decapitated and the heads fixed in a 10% formalin solution for 48 hours. An upper and a lower quadrant were used to evaluate the number and extent of carious lesions by the grinding and observation procedure (Shaw, Schweigert, McIntire, Elvehjem and Phillips, '44). The other quadrants were dehydrated and imbedded in methyl methacrylate (Sognnaes, '47). A sagittal anteroposterior ground section was made of each jaw for detailed study of the carious lesions.

In the second experiment various isocaloric modifications of ration 100 were made to alter the particle size of its components and to alter the texture and the consistency of the resulting rations. The approximate energy value of that aliquot of each ration listed in table 1 was 409 cal., as calculated with the Atwater standards of 4, 9 and 4 cal. per gram of carbohydrate, fat and protein, respectively. No accurate energy value could be assigned for the 4 gm of liver concentrates in the above aliquot of each ration.

In the first series of rations (120, 130 and 140) progressive increases in the fat content were made by the isocaloric substitution of 4, 8 or 12 gm of lard for 9, 18 or 27 gm, respectively, of each 67 gm of sucrose in the basal ration. These levels of substitution were chosen so that two would be lower and the third slightly higher than the lower level at which a reduction in tooth decay was observed by Schweigert et al. ('46). In this way a closer estimate could be made of the lowest level of fat and the highest level of sucrose at which a substantial reduction in the average number and extent of carious lesions per cotton rat could be produced. Marked differences in the consistency of the ration resulted with isocaloric substitution. A progressive decrease in the

granular, particulate texture which was characteristic of ration 100 and a progressive increase in the greasiness and plasticity of the rations was observed with each increase in lard content and decrease in sucrose content, until ration 140 had the consistency of a somewhat granular, malleable dough.

The particle size and consistency of rations 100, 130 and 140 were altered by replacement of the granulated sucrose, the only coarse ingredient, by finely powdered sugar in order to give rations 104, 134 and 144. Ration 104 was extremely fine and almost dusty in nature, and its particles did not adhere to each other. The particles in rations 134 and 144 had an increasing tendency to adhere to each other, but did not feel as greasy and were not as pasty or malleable as their counterparts (130 and 140) prepared with granulated sucrose. On the basis of appearance and touch, ration 144 could be described as being roughly comparable in consistency to ration 120 which contained much less fat.

The consistency of rations 100, 120, 130 and 140 was also altered by the incorporation in the rations of cellu flour, which has been shown independently to have no effect on the initiation and development of carious lesions in the cotton rat (Howell, Schlack, Taylor and Berzinskas, '48). The presence of cellu flour materially decreased the greasy texture of the rations and maintained them in a more particulate form due to the greater dispersion of the fat, but did not alter the relative distribution of calories from fat and carbohydrate nor the relation of calories to minerals and vitamins.

In another series of rations the sucrose was entirely replaced by either fine or coarse dextrin (ration 101 and 102, respectively). The fine dextrin had particles which were sufficiently small to pass readily through a 60-mesh sieve. The coarse dextrin was composed of particles of varying size: 30% were retained on a 20-mesh sieve and an additional 50% were retained on a 60-mesh sieve, while the remainder passed through both. Ration 101 had a consistency and

powdery nature similar to those of ration 104, where sucrose had been replaced by powdered sugar. Ration 102 was much coarser than ration 100 and was outstandingly different from other rations studied because of the extensive mastication necessitated in the consumption of the coarse and flinty dextrin particles.

The litters of cotton rats used in the second experiment were distributed as equally as possible among the control and experimental groups. All animals were continued on the dietary regimen for 14 weeks, when they were sacrificed and the heads fixed in 95% ethanol. The number of carious lesions and the amount of tooth substance involved in each lesion were evaluated by the grinding and observation procedure of Shaw, Schweigert, McIntire, Elvehjem and Phillips ('44).

## RESULTS

A series of photomicrographs of ground sections is presented in figures 1 to 6, inclusive, to indicate certain types and stages of the carious lesions which develop in the molars of cotton rats. A description of the anatomical characteristics of the molar teeth and of the readily visible carious lesions accompanies the illustrations.

### *Accumulation of food debris*

The sulci (grooves) usually contain some debris, which differs widely in amount, color, histologic appearance and readiness with which the particles can be removed with a fine probe. The presence of such material does not appear to be associated necessarily with fracture of the cusps nor with an early destruction by carious lesions of adjacent tooth structures. Frequently sulci with extensive tooth decay contain little foreign matter, as in figures 2 and 5; certain sulci may be full of material and yet be accompanied by little or no evidence of tooth decay, as in figure 1. When the grooves are as narrow and deep as those in the cotton rat, one would

scarcely expect any appreciable interchange of the materials in the sulci, especially in the case of fibrous, or tightly wedged, particles. Since some particles removed from the sulci during the examination for carious lesions resembled the stock ration, or fragments of wood shavings, to neither of which the rats had had access since they were weaned, some particles probably were forced into the sulci prior to weaning.

The accumulation of material was rare on the surfaces of the molar teeth which face the cheek, the tongue or the adjacent teeth. At times a light brown, hard, "candied" residue was observed on the third of the molar crowns nearest the gingiva, and was especially noticeable on first molars. This accumulation was not associated with carious lesions of these surfaces and was not sufficiently extensive to cause any detectable damage to the supporting structures of the teeth during the length of the experimental period. Soft, pasty residues were observed only on the smooth surfaces of the molars in cotton rats from which the major salivary glands had been removed, and even then did not appear to be associated with the initiation of lesions (Shaw and Weisberger, '49).

### *Position of carious lesions*

The most common place where carious lesions occurred in the molar teeth was at the bases and on the anterior and posterior surfaces of the sulci (figs. 2 and 3); the next most common site was on the surfaces (figs. 2 and 6) in contact with the adjacent teeth. Usually the point of origin of the lesions was a considerable distance below the occlusal surface. The earliest lesion detected consisted of an area in the enamel which was soft and pigmented. The region involved in these early lesions differed greatly in size, varying from a small area of penetration to rather wide, diffuse areas of invasion. These lesions occurred prior to any fracture or detectable mechanical injury of the enamel. Lesions on the tooth surfaces which face the cheek and tongue were observed

with extreme rarity. As yet, no subgingival lesions have been detected. The exposed dentin on the occlusal surface of the molars was not readily susceptible to tooth decay. The latter type of lesion did occur frequently in third molars in the period immediately following their eruption into the oral cavity and is particularly extensive in cotton rats, where the process of eruption of the third molars is unusually late (fig. 1).

The rate of progress of carious lesions was found to be very rapid. Well-developed lesions of the enamel with slight dentin penetration were present in highly susceptible animals as early as the 5th week on experiment. Almost complete destruction of the molar crowns in all quadrants was observed in some susceptible animals by the 10th week.

Fracture of cusps did not occur prior to initial lesions in the molars of cotton rats maintained on ration 100. When large areas of molar cusps had been undermined by the carious process the forces of mastication were sufficient to produce secondary fracture with loss of fragments of enamel and dentin.

#### *Alterations in particle size and consistency of rations*

The effects of the various purified rations upon the average number and average extent of carious lesions during the 14-week experimental period are presented in table 2. Ration 120, in which 9 of each 67 gm of sucrose in ration 100 had been replaced by 4 gm of lard, did not result in any appreciable difference in the average number and average extent of carious lesions from those values observed for the litter mates fed ration 100. The feeding of ration 130, in which an additional 9 gm of sucrose had been replaced by 4 gm of lard, resulted in a slightly lower average number and average extent of carious lesions than were found in the control litter mates. When ration 140 was fed, where a total of 27 gm of each 67 gm of sucrose in ration 100 had been isocalorically replaced by lard, there was a strikingly lower average number and average extent of carious lesions than in the



TABLE 2

*The average number and extent of carious lesions observed in cotton rats maintained for 14 weeks on various purified rations*

RATION NO.	NUMBER OF COTTON RATS	AVERAGE NUMBER OF CARIOUS LESIONS	S.E.M. <sup>1</sup>	C.R. <sup>2</sup>	AVERAGE EXTENT OF CARIOUS LESIONS	S.E.M. <sup>1</sup>	C.R. <sup>2</sup>
100	14	24.2	1.8	> 0.3	66 +	8 +	> 0
120	12	24.9	1.4		66 +	8 +	
100	29	27.3	1.1	> 2.6	79 +	5 +	> 3.7
130	27	23.4	1.0		55 +	4 +	
100	15	24.8	1.2	> 4.9	65 +	7 +	> 4.9
140	15	15.9	1.3		27 +	4 +	
100	11	27.0	1.5	> 0.7	78 +	7 +	> 0.9
104	8	28.9	2.1		88 +	9 +	
130	9	25.0	0.7	> 0.4	66 +	4 +	> 1.5
134	7	24.1	2.3		52 +	8 +	
140	8	15.0	1.3	> 0.5	22 +	4 +	> 0.4
144	9	16.0	1.5		25 +	6 +	
100	6	26.5	1.2	< 0.7	85 +	8 +	< 0.3
100A	8	27.9	1.7	×	88 +	7 +	×
100B	7	26.0	1.5	< 0.3	89 +	9 +	< 0.3
120	9	23.7	1.4	> 0.8	59 +	10 +	> 0.4
120B	7	21.9	2.0		53 +	11 +	
130	9	20.8	1.9	> 0.3	40 +	8 +	> 0.4
130A	7	22.0	2.8		46 +	11 +	
130	8	23.8	1.0	> 0.2	55 +	4 +	> 0.6
130B	8	23.4	1.8		51 +	6 +	
140	8	15.3	1.9	> 0.7	23 +	3 +	> 1.1
140A	9	17.1	1.7		29 +	4 +	
100	9	25.9	1.1	< 9.8	81 +	4 +	< 13.2
101	8	10.4	1.2	×	23 +	2 +	×
102	8	4.4	0.9	< 4.0	9 +	2 +	< 6.2

<sup>1</sup>Standard error of mean.

<sup>2</sup>Critical ratio.

litter-mate controls. On the basis of experimental data, the critical level of fat at which occurred a substantial reduction in the average number and average extent of carious lesions appeared to lie between 13.8 and 19.1%, with no appreciable protective effect at a level of 9.1%. Or, to express the relationship in terms of sucrose, no substantial reduction in the average number and average extent of carious lesions occurred until the sucrose level was reduced to 52.1% or less by isocaloric replacement with fat.

The substitution of finely powdered sugar for the granulated sucrose in rations 100, 130 and 140 did not alter the initiation and development of carious lesions in the cotton rat. Similarly, the addition of indigestible material in the form of cellu flour to rations 100, 120, 130 and 140 did not affect the initiation and development of carious lesions.

The complete replacement of the granulated sucrose in ration 100 by fine or coarse dextrin resulted in striking reductions in the average number and average extent of carious lesions, as reported previously (Shaw, Schweigert, McIntire, Elvehjem and Phillips, '44). The ingestion of ration 102 containing coarse dextrin resulted in an extensive degree of attrition and a widespread initial fracturing of the molar cusps, with a resulting modification of the normal structural characteristics of the grooves. Those few carious lesions in the cotton rats fed the coarse dextrin diet were most frequently found on the dentin surfaces exposed by these fractures and not on the few remaining intact enamel surfaces. The effect of the replacement of granulated sugar by coarse dextrin appeared to entail not solely a replacement of the form of carbohydrate but, in addition, a modification of the structure of the teeth and, possibly in this way, a modification of caries susceptibility.

No more attrition or fractures occurred in the molars of cotton rats fed the fine dextrin diet 101 than in those of their litter mates fed ration 100. The carious lesions produced in cotton rats fed diet 101 were characteristic of early lesions in control animals and appeared to progress ex-

tremely slowly. Since cotton rats disliked the finely powdered dextrin diet sufficiently that they refused or spilled a high percentage of the amount offered, an insufficient quantity was eaten to allow normal growth and well-being. Undoubtedly this has been a complicating factor in the initiation and development of carious lesions on this dietary regimen. A comparably drastic caloric restriction has been found to be associated with a greatly reduced incidence and extent of carious lesions in the cotton rat (Shaw, '48). Thus the feeding of diets where the sucrose had been replaced by either coarse or fine dextrin resulted in complications which could not be attributed to the replacement of sucrose per se.

### *Rates of growth*

The average rates of growth during the experimental periods and the average final body weights for the various groups of cotton rats in these investigations were normal, with one exception. The cotton rats fed the fine dextrin diet grew at a much slower rate and attained much lower final body weights than the litter mates fed ration 100. Rations 104, 134 and 144, in which the sucrose had been replaced by powdered sugar, allowed the same rates of growth and final body weights as their counterparts, 100, 130 and 140. The fine particle size of the powdered sugar materially affected the over-all particle size and consistency of the rations but, except for the occasional animal fed ration 104, the rations containing powdered sugar were not disliked by the cotton rats to such an extent that reduced amounts were eaten. Despite the comparable particle size of the components of rations 104 and the fine dextrin ration, 90% of the cotton rats offered the former ration ate it and did well, while all the animals offered the fine dextrin ration ate little and grew poorly. Part of the difference in the acceptance of the two rations seemed to be due to the ability of the corn oil to cause the particles of powdered sugar to adhere loosely, whereas the same amount of corn oil was insufficient to cause any binding of the fine dextrin particles. The addition of

various amounts of cellu flour to the diets did not consistently alter the rates of growth of the cotton rats in these experiments. Similarly, supplements of cellu flour did not increase the survival rate of cotton rats fed purified rations, as has been suggested previously by Howell, Schlack, Taylor and Berzinskas ('48). Survival in the above experiment seemed to be dependent to a great extent upon the unpredictable presence or absence of intercurrent infections in the animal quarters.

#### DISCUSSION

Histologic studies of the molar teeth from cotton rats which had been maintained on purified ration 100 failed to indicate the presence of any mechanical injury of the caries-susceptible enamel surfaces previous to the initiation of carious lesions. Since the grooves in cotton rat molars are extremely narrow, and since most early carious lesions were initiated toward the bases of the sulci or on the surfaces adjacent to other teeth, the caries-susceptible areas would appear to be well protected from physical trauma. The replacement of sucrose, the only coarse constituent in rations 100, 130 and 140, which require little mastication, by finely powdered sugar resulted in rations with very finely subdivided particles requiring no appreciable amount of mastication. The fact that this reduction in particle size did not alter the initiation nor the rate of development of carious lesions further indicated that these conditions are not dependent upon preliminary injury to the enamel surfaces.

Tests on the replacement of the soluble carbohydrate, sucrose, by a less soluble one, dextrin, were inconclusive because of certain uncontrolled variants: partial caloric restriction in the case of replacement by fine dextrin, and extensive abrasion and fracture in replacement by coarse dextrin. One point demonstrated by these studies was that fracture of the tooth surfaces in the cotton rat is not necessarily associated with a high degree of initiation and development of carious lesions; extensive fractures could be associated with a lower incidence of dental caries than when

the same diet was fed in a form not conducive to mechanical injury of the teeth. In this regard there is a clear-cut difference between the early experiments with the common laboratory rat using the coarse particle, Hoppert-Webber-Canniff diet and investigations in the cotton rat using purified rations.

The mechanism by which isocaloric replacement of dietary carbohydrate caused reductions in the initiation and development of carious lesions needs considerable further investigation. Once the ratio of fat to sucrose in a ration had been increased until a slight reduction in the incidence and extent of carious lesions occurred, a small further increase in this ratio resulted in a further rather considerable reduction in the dental caries experience. The ration (140) with the highest fat:sucrose ratio contained 44.9% by weight of the digestible components as sucrose and had a fat content of only 19.1%. Yet cotton rats fed this ration had a 36% lower average number of carious lesions and a 59% lower average extent of carious lesions than the control litter mates. The various fat:sucrose ratios tested had the same effect upon the initiation and development of carious lesions regardless of whether the carbohydrate in the ration was coarse granulated sucrose or fine powdered sugar or whether large amounts of cellulose flour had been added. By both procedures, rations with increasingly higher fat:sucrose ratios could be produced in more particulate, somewhat non-greasy forms instead of greasy masses through the distribution of the fat over a greatly increased number of particles, both with and without a dilution of the nutrients by an inert, non-nutritious material. The lack of a demonstrable relationship between the consistency of the ration, the distribution of the fat over a large or small number of particles, and the initiation and development of carious lesions in the cotton rat strongly suggests that the increasing fat content of the diet was not effective in reducing the dental caries attack rate through a mechanical alteration in the form of the diet or its constituents.

## SUMMARY

1. Carious lesions in cotton rats maintained on purified rations were most common deep in the sulci and on the proximal surfaces of the molar teeth. Occasional lesions were found on exposed dentin of the occlusal surfaces, but almost none on surfaces which face the cheek or tongue.

2. Histologic examination of ground sections of cotton rat jaws indicated that the carious lesions in the sulci and on the proximal surfaces began on intact enamel surfaces well below the occlusal plane. There was no evidence of fracture previous to the initiation of carious lesions except in those cotton rats fed a ration in which sucrose had been replaced by coarse dextrin.

3. The isocaloric replacement with lard of 9 gm of each 67 gm of sucrose in the basal ration did not alter the number nor the extent of carious lesions in the cotton rats. The isocaloric replacement by lard of 18 gm or 27 gm of each 67 gm of sucrose in the basal ration resulted in increasingly significant reductions in the dental caries attack rate.

4. The complete replacement of the sucrose in rations with varying fat content by finely powdered sugar altered the particle size of the rations greatly but did not result in any alteration in the average number nor in the average extent of carious lesions.

5. The addition of large amounts of crude indigestible fiber in the form of cellu flour to rations with varying fat content greatly altered the consistency of the rations but did not alter the average number nor the average extent of the carious lesions.

6. Replacement of sucrose in the control ration by fine or coarse dextrin resulted in highly significant reductions in the dental caries experience. These reductions could not be attributed entirely to the simple replacement of a soluble carbohydrate by a less soluble or insoluble one.

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## EXPLANATION OF FIGURES

Figs. 1-6 Ground sections of cotton rat jaws in the vertical antero-posterior plane, imbedded in polymerized methyl methacrylate. The large number in the lower right corner of each photograph is the figure designation. The small characters on the upper margin of each photograph are from the scoring code to designate the sulci immediately below them, and will be referred to in the description of each figure in order to locate the lesions. The letter A is placed on each photograph to designate the anterior aspect. In all cases the occlusal plane is at the top of the photograph.

Fig. 1 A lower left jaw of a cotton rat which had been maintained on the basal purified ration for 8 weeks. The narrow and extremely deep sulci, the rather thin border of enamel, and the packing of debris in the sulci can be seen. Examination of the sulci in the first and second molars discloses only small enamel lesions which are not readily visible at this magnification. In sulci 37 and 39 of the third molar extensive lesions can be seen. The lesion in sulcus 37 appears to have begun on the exposed dentin of the occlusal surface. The lesion in sulcus 39 may have begun on the posterior enamel surface or on the dentin of the occlusal surface. In both cases there has been a considerable loss of the tooth structures above the lesion.  $\times 15$ .

Fig. 2 A lower right jaw of a cotton rat which had been maintained on the basal purified ration for 14 weeks. The carious lesions in this jaw are more frequent and more extensive than those in figure 1. In sulcus 24 of the first molar there is a lesion which appears to have begun on the posterior surface and progressed until the portion of the cusp undermined by the lesion has been fractured under the pressure of mastication. An extensive lesion has occurred in sulcus 28, with a penetration of the posterior surface until fracture has occurred. On the anterior side of sulcus 28 there is a small pigmented area in the enamel indicative of an early lesion. On the posterior surface of the first molar there is a small but rather rapidly progressing enamel lesion which has not reached the dentin. In the second molar there is a small on the anterior surface or in sulcus 32 or in both. The exact origin cannot be determined since fracture of the cusp has occurred, obliterating the original cusp and the sulcus. In sulcus 34 there is a large lesion on the posterior surface which has penetrated into the dentin but which has not yet resulted in any loss of tooth structure due to this undermining. In sulcus 40 of the third molar there is an area of involved enamel on both the anterior and posterior surfaces directly opposite each other. The carious area on the posterior surface has penetrated into the dentin, while the lesion on the proximal surface has not progressed completely through the enamel. This jaw illustrates the customary occurrence of all stages of lesions in one jaw, all of which can be evaluated with reasonable accuracy in so far as their origin is concerned except the lesion involving both sulcus 32 and the anterior surface of the second molar.  $\times 15$ .

Fig. 3 A portion of a lower left third molar. On the posterior surface of sulcus 39 near the occlusal surface is a small lesion with a loss of enamel structure and dentin penetration. Somewhat deeper in the sulcus are two small pigmented enamel lesions opposite each other on the anterior and posterior surfaces. Toward the bottom of the sulcus there is an area of penetration in the dentin which appears to have been associated with some lesion in which the enamel area involved is not represented in the plane of this section.  $\times 60$ .

Fig. 4 A lower right third molar and the posterior surface of the second molar. On the anterior surface of the third molar a wide, pigmented enamel lesion is visible. The posterior surface of sulcus 40 has a carious area with a wide enamel and a deep area of dentin involvement which has not been complicated by fracture in mastication. A wide area of enamel and a deep area of dentin have been involved in the carious process.  $\times 45$ .



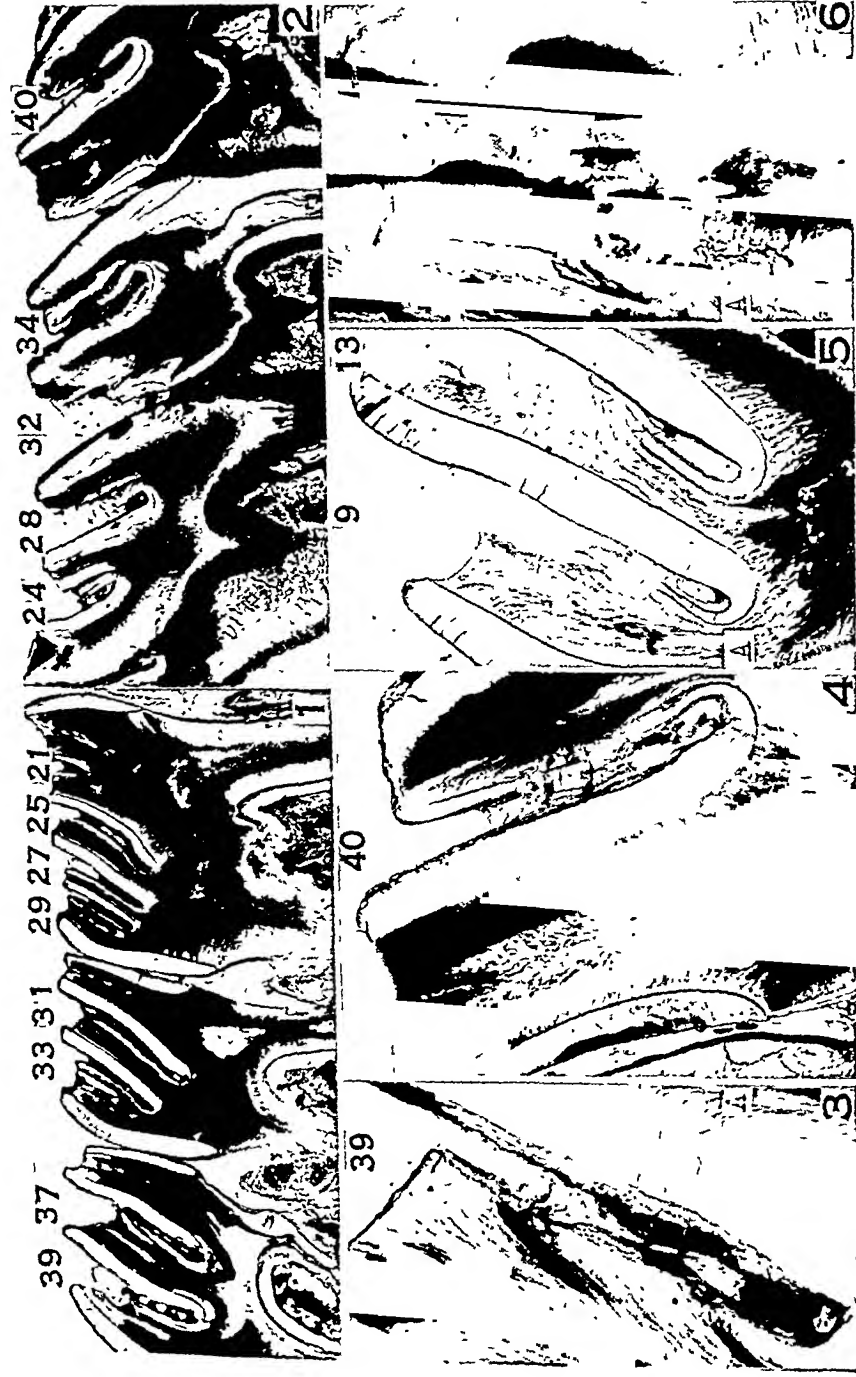


Fig. 5 An upper left second molar. In sulci 9 and 13 are extensive lesions on the anterior surface in which fracturing of the cusps has also occurred, but after the initiation of the lesions. The intact dentin at the occlusal surface of each sulcus would seem to indicate that these lesions did not begin on the dentin surface. The irregular areas on the posterior surface of the second molar and the proximal surface of the third molar are artefacts due to the inclusion of air bubbles when the block containing this jaw was being mounted on the slide.  $\times 45$ .

Fig. 6 The proximal surfaces of lower right first and second molars. A cautious lesion is shown on both the posterior surface of the first molar and on the anterior surface of the second molar. In the former lesion there has been a definite penetration of the dentin.  $\times 60$ .



# DETERMINATION OF SOME ESSENTIAL AMINO ACIDS IN SEVERAL UNCOOKED AND COOKED MEXICAN FOODSTUFFS

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While in other countries meat, eggs, and milk are the principal sources of protein in the diet, in Mexico they are not commonly used, because of their excessive cost or because of traditions very firmly established among the people. The usual Mexican diet among the poorer classes consists chiefly of corn, beans and pulque, supplemented with some vegetables, among which chiles and, in the city of Mexico, rice are outstanding.

The present study has as its purpose to add to our knowledge the nutritive value of these foodstuffs. Data concerning their caloric, vitamin, and mineral content may be found in the articles of Cravioto et al. ('45b) and Cravioto and Miranda ('47) but little is known of the quantities they contain of the so-called essential amino acids. Among the first papers of this kind relating to Mexican foods is that of Giral and Cravioto ('41) on the threonine content of corn, but there is no information available concerning food products prepared for consumption. For this reason the present study also considers the composition of tortillas, cooked beans, and cooked rice. It is the belief of the authors that the basic principle for judging whether a source of

proteins is suitable or not is knowledge of the quality of these proteins; that is to say, whether the necessary quantities of essential amino acids are present, since according to some authors (Elvehjem, '46; Krehl et al., '46) a diet rich in poor-quality protein can do more damage than a diet with less protein but with a balanced content of amino acids.

## EXPERIMENTAL PROCEDURE

### *Samples*

A sample was taken of the white corn (*Zea mays* L.) from which tortillas are made following the method commonly used in Mexico as reported by Cravioto et al. ('45a), which is treatment with  $\text{Ca}(\text{OH})_2$  and heating. Starch was removed from these samples by salivary digestion according to the method of Doty ('41). For the beans, the variety used was one of the most common in Mexico, palacio beans (*Phaseolus vulgaris* L.), a part of which were cooked with water by boiling three to 4 hours at atmospheric pressure to obtain what is called in Mexico "frijoles de olla" (stewed beans). As with the corn, the starch was removed from both the raw and cooked beans by a modification of the method of Doty. The rice used was the unpolished white product such as is consumed in Mexico, a part of which was cooked using the technique for the preparation of "arroz blanco," as follows: (a) the rice is washed with hot water which is discarded and then with cold water which is discarded too; (b) the rice is drained and heated with oil or lard for 5 to 10 min., the excess of oil being removed; (c) the rice is covered with water and cooked for half an hour, adding salt to taste and water as necessary. For analysis the samples of raw and cooked rice were treated with ether in the Soxhlet extractor for 16 hours to remove the fat. All the grains were finely ground with mortar and pestle and dried *in vacuo* over  $\text{P}_2\text{O}_5$ . A sample of pulque "de tinacal" (factory pulque) was taken and the analysis begun without any special previous treatment.

### *Nitrogen analysis*

For the corn, tortillas, raw beans and cooked beans, the micro-Kjeldahl method was used on duplicate samples of 10 to 20 mg dried over  $P_2O_5$ . For the raw and cooked rice the method of Kjeldahl was used on samples of 0.5 gm dried as in the previous case. The pulque was analyzed by the Kjeldahl method, using samples of 25 ml.

### *Preparation of samples for analysis*

Hydrolysates were made on duplicate samples of 100 mg of corn, tortillas, and raw and cooked beans, and of 1.0 gm of raw and cooked rice, each with 10 ml of 10% HCl, by autoclaving 10 hours at 15 lb. for the determination of all amino acids except tryptophan. For this last determination hydrolysis was carried out at 15 lb. for 10 hours with 5 ml of 5 N NaOH on samples of 50 mg for corn, tortillas and raw and cooked beans and of 0.5 gm for raw and cooked rice, the last two with 10 ml of 5 N NaOH. For the analysis of pulque, 500 ml were hydrolyzed with HCl added to give a final concentration of 10%. To make the alkaline hydrolysate, 20 gm of NaOH were added to 100 ml of pulque, giving a concentration of approximately 20%. Both samples were hydrolyzed at 15 lb. for 10 hours. The samples were all neutralized and the pH adjusted to 6.8 after elimination of the humin. They were diluted as desired.

### *Amino acid analysis*

The microbiological method of Stokes et al. ('45) was employed for the determination of amino acids, utilizing *Streptococcus faecalis* and *Lactobacillus delbrückii* LD5. In some cases (beans, cooked beans, rice, cooked rice, and pulque) 2.5 ml of the basal medium were used instead of 5 ml, with the same dilution of the inoculum. The standard solutions were made at the same concentration as in the original method; the final volume in the tubes was 5 ml. The acid produced during incubation was titrated with 0.025

TABLE 1  
*Amino acid content of the foods analyzed*

SUBSTANCE	NITROGEN PER CENT OF DRY- WEIGHT	PER CENT, CALCULATED TO 16% NITROGEN, ON DRY BASIS								
		Lysine	Trypto- phan	Histi- dine	Phenyl- alanine	Leu- cine	Threo- nine	Methio- nine	Valine	Arginine
Corn, defatted <sup>1</sup>	6.91	2.5	0.6	2.3	4.4	19.6	4.7	1.9	5.4	4.4
Tortilla, defatted <sup>1</sup>	6.06	2.2	0.4	1.7	4.3	18.9	3.5	1.7	5.4	3.7
Beans, defatted <sup>1</sup>	6.69	6.0	1.0	2.5	5.4	13.2	5.0	0.7	5.3	4.7
Beans (cooked), defatted <sup>1</sup>	6.83	5.7	1.0	2.4	5.3	11.8	4.8	0.7	5.1	4.7
PER CENT, CALCULATED TO 16.8% NITROGEN, ON DRY BASIS										
Rice, defatted	1.32	3.3	1.5	2.1	4.6	9.7	4.5	1.9	6.7	6.3
Rice (cooked), defatted	1.17	3.3	1.7	1.8	4.8	9.5	4.1	2.0	6.8	5.8
PER CENT, CALCULATED TO 16% NITROGEN, ON DRY BASIS										
Whole egg proteins <sup>2</sup>		7.2	1.5	2.1	6.3	9.2	4.9	4.1	7.3	6.4

<sup>1</sup> The starch was removed by salivary digestion.

<sup>2</sup> Data from Mitchell and Block ('46).

N NaOH except in the determinations of phenylalanine, in which case 0.05 N NaOH was used. Recovery tests were carried out by adding known amounts of amino acids to samples of corn and tortilla prior to hydrolysis, using 2.5 ml of basal medium per tube.

## RESULTS

The amino acid content of the foods analyzed, with the exception of the pulque, is reported in per cent of total protein as referred to dry matter. As recommended by the United States Department of Agriculture ('31), the factor 6.25 was used to calculate the protein content of the corn, tortillas and beans (cooked and uncooked), and the factor,

TABLE 2  
*Amino acid content of the pulque*

NITROGEN GM PER 100 ML	AMINO ACID CONTENT, IN MG PER 100 ML								
	Lysine	Tryptophan	Histidine	Phenylalanine	Leucine	Threonine	Methionine	Valine	Arginine
0.14	16.2	2.7	4.7	11.2	10.5	6.4	0.7	6.6	10.9

5.95 for the raw and cooked rice. The results are reported in table 1 with the exception of those relating to the pulque, which appear in table 2, where the amino acids are expressed as milligrams per 100 ml. We wish to point out that in the case of rice cooking causes an appreciable loss of nitrogen, probably because the wash waters which were discarded removed some nitrogen compounds. In terms of dry matter, uncooked rice and the same rice cooked contained 1.32% and 1.17% of nitrogen, respectively.

In table 3 there is a comparison of the data that we have obtained for whole corn and rice, on the basis of 16% of nitrogen. We have not found any reports on the amino acid composition of the bean studied (*Phaseolus vulgaris*). There are only references to the  $\alpha$  and  $\beta$  globulins in the black beans of the Mayas, in which Jones et al. ('38) found some amino acids.

TABLE J  
*Comparison of some values reported in the present paper (whole corn and rice) with others observed by different authors*  
 Per cent, calculated to 16% nitrogen, on dry basis

AMINO ACID	WHOLE CORN				RICE			
	Values in the present work	Values from literature		Values in the present work	Values from literature		Block and Bolling <sup>1</sup>	Block <sup>4</sup>
		Block and Bolling <sup>1</sup>	Horn et al. <sup>2</sup>		Schweiger <sup>3</sup>	Horn et al. <sup>2</sup>		
Lysine	2.5	2.5	2.3	3.1	2.3, 2.3	2.8	3.2	3.2
Tryptophan	0.6	0.6		1.4			1.3	1.3
Histidine	2.3	2.4	1.9	2.0	2.4, 2.2	2.0	1.5	1.5
Phenylalanine	4.4	4.5	5.2	4.4	4.3	4.8	6.2	6.7
Leucine	19.6	21.5 ± 2.4		9.2	9.6		7.7	9.0
Threonine	4.7	3.6	3.9	4.3	3.1, 3.9	3.5	3.9	4.1
Methionine	1.9		1.4	1.8		1.4	3.4	3.4
Valine	5.4	4.6 ± 0.7	5.3	6.3	5.2		6.4	6.3
Arginine	4.4	4.0	4.7	6.0	4.7	8.2	7.2	7.2

<sup>1</sup> Block and Bolling ('45).

<sup>2</sup> Horn et al. ('46, '47a, '47b, '47c, '48a, '48b, '48c).

<sup>3</sup> Schweiger ('48).

<sup>4</sup> Block ('45).



Recoveries of known amounts of amino acids added to substances prior to hydrolysis are quantitative within  $\pm 5\%$ . The results are reported in table 4.

Generally speaking, our data on the amino acid content of whole corn and rice agree with those reported by several investigators. Our data on threonine and methionine in whole corn and on arginine, methionine and phenylalanine in rice are not in accord with those of some authors. We believe that the disagreements are due to some extent to the different methods employed for the analysis of the amino

TABLE 4  
*Recovery of amino acids added to proteins prior to hydrolysis*  
(Milligrams per gram of dried material)

AMINO ACID	SAMPLE	CONTENT	ADDED	TOTAL	FOUND	PER CENT RECOVERY
Threonine	Tortilla	12.7	15.0	27.7	26.5	96
Valine	Tortilla	19.9	20.0	39.9	40.7	102
Phenyl- alanine	Tortilla	16.0	15.0	31.0	30.0	97
Leucine	Tortilla	71.9	75.0	146.9	145.5	99
Methionine	Tortilla	6.6	5.0	11.6	11.8	102
Arginine	Tortilla	14.0	15.0	29.0	30.2	104
	Corn	19.7	15.0	34.7	36.8	105
Histidine	Tortilla	6.5	5.0	11.5	11.3	98
	Corn	10.0	5.0	15.0	14.3	95
Lysine	Corn	10.4	10.0	20.4	19.4	95

acids. On the other hand, possibly they may be due to the differences that Doty et al. ('46) observed in the amino acid content of corn because of the influences of genetic factors, and also to the fact, observed by Kik ('41), that the use of fertilizers affects the amino acid content of rice.

#### DISCUSSION

If we accept the criterion of Mitchell and Block ('46), it is important to evaluate the quality of a protein with respect to its amino acid content by comparing it with whole egg. Experiments, based on growth in the rat, carried out by

these authors show whole egg protein to be perfectly balanced in amino acids. For this reason, at the bottom of table 1 are shown the figures reported by Mitchell and Block for whole egg. In this manner we can calculate the biological value of the foods analyzed in spite of the lack of data concerning isoleucine, tyrosine, and cystine, the last two being considered semi-essential by Block and Bolling ('44). Practically the same criterion has been upheld by Giral ('43), who compared the quantity of amino acids furnished by various foods with that furnished by eating two eggs daily, which he considers to contain sufficient essential amino acids.

According to the point of view of Mitchell and Block, then, it can be stated that corn is more deficient in lysine than in tryptophan, and that beans are deficient in methionine. Rice is also deficient in lysine but not in tryptophan. A deficiency in methionine can also be seen in corn and rice. The other amino acids deviate less from the quantities in which they appear in whole egg proteins. It must, however, be borne in mind that such a comparison can only be a rough one, because of the differences in the methods of amino acid analysis employed by Mitchell and Block for the whole egg and by the present authors for the foodstuffs we analyzed.

From the results obtained it can be inferred that beans can compensate to a certain degree for the lack of lysine in corn and rice and, on the other hand, that rice can compensate for the deficiency of tryptophan in the other two. But on a diet of beans, corn, and rice combined in the proportions in which our people consume these foods, the deficiencies of lysine and tryptophan would not be compensated for. From the information obtained from various surveys by Anderson et al. ('46) and Calvo et al. ('46) it is known that in the Mexican diet tortillas predominate in quantity over beans and rice. According to Miranda ('47), the daily intake of the average Mexican is 216.2 gm of corn, 22.5 gm of beans, and 11.6 gm of rice. This diet is not balanced with respect to lysine and tryptophan.

The results obtained with the pulque (see table 2) should be considered tentative. Since the nitrogen in this drink can vary within wide limits, determinations on a large number of samples are necessary to obtain definite figures. We can, however, say that the results show that considerable amounts of amino acids, particularly lysine, must be obtained from the large quantities of pulque that certain sectors of the population drink.

The changes produced by heat processing in different foods with respect to transformations in the composition of amino acids have been brought out as much by experiments with rats as by chemical and microbiological analysis. There is the work of Olcott and Fontaine ('41), who found that by autoclaving cottonseed meal its biological value is lessened and that by the addition of lysine the value returns to normal. Block et al. ('46) found in the same way that the decrease in biological value of a cake mix of several foods by heating is due to the transformations that occur in the lysine constituent. Greaves et al. ('38) observed that casein heated at 140°C. for 30 min. loses lysine and histidine without damage to cystine, tyrosine and tryptophan. Block et al. ('34) found by chemical analysis that the lysine content does not change materially in casein heated at 150°C. Mitchell and Block ('46) noted slight changes in the amino acid content of a corn-oats-rye mixture submitted to the procedure of "gun explosion" to obtain "rolled oats." These authors reported changes principally in the lysine (9.5%), valine (11.2%), and isoleucine (12.6%) content. However, they consider that there is not fundamentally a change in the composition of the mixture. Patton et al. ('48) analyzed casein by microbiological methods before and after heating it with glucose by reflux for 24 hours and found losses in lysine (26%), arginine (30%), and tryptophan (39%).

The present authors found (see table 1) changes resulting from the cooking of beans under the above conditions that amounted to only 9% in leucine and about 8% in lysine. Rice when cooked suffers its greatest change in its trypto-

phan content (13.3%). We consider that these changes do not essentially alter the composition of the analyzed foods from the standpoint of nutrition, notwithstanding that they are slightly beyond the range of error of the amino acid assay ( $\pm 5\%$ ).

In the same manner we can see the variations that corn undergoes in passing from the raw state to that of the tortilla. These changes are principally characterized by losses in tryptophan (30%), threonine (26%), histidine (25%) and arginine (16%), and they go far beyond the range of error of the method for the amino acid assay. We did not find in the literature any reports on tortillas themselves nor on other foods that had been treated in the same way, and we believe that the observed changes, though they may seem excessive, are not produced by simple heating alone since the preparation of the tortilla involves the heating of corn with a  $\text{Ca}(\text{OH})_2$  solution for almost an hour and further cooking in the "comal" (hot plate). The changes in amino acids may be due not only to the action of the heat but in part to the action of the hot  $\text{Ca}(\text{OH})_2$  solution, possibly to partial racemization of the proteins such as Levene and Bass ('28) observed in casein heated with different NaOH solutions.

#### SUMMARY AND CONCLUSION

1. Nine essential amino acids were determined in corn, tortillas, beans (cooked and uncooked), rice (cooked and uncooked), and pulque, using the microbiological method.

2. Compared with whole egg, the corn, tortillas, and uncooked rice were deficient in lysine. Corn and tortillas were also deficient in tryptophan, and beans in methionine.

3. In preparing tortillas considerable changes occur in the tryptophan, histidine, threonine, and arginine content of the corn as determined by microbiological analysis, using *Streptococcus faecalis*. Beans when cooked show practically no such changes. Similarly, rice remains essentially the same on being cooked.

4. Appreciable quantities of amino acids, especially lysine, were found in pulque.

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# THE VALUE OF RUTIN AND QUERCETIN IN SCURVY

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## ONE FIGURE

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Rutin, the rhamno-glucoside of quercetin, became the subject of study in this laboratory following its recent large-scale isolation from buckwheat (Eskew, Phillips, Griffin, Shaines and Aceto, '48). The value of flavones and flavonols in the treatment of hemorrhagic diathesis has been summarized in Nutrition Reviews ('43, '44) and the British Medical Journal ('47). Bentsáth, Rusznyák and Szent-Györgyi ('36) reported that a beneficial effect of "citrin" on vascular permeability could be demonstrated experimentally in guinea pigs. These authors showed that guinea pigs on a scorbutogenic diet could be kept alive longer with a daily supplement of 1 mg of "citrin" than without it. From this evidence they postulated the existence of vitamin P, a substance other than ascorbic acid required by scorbutic guinea pigs.

In a later article Bruckner and Szent-Györgyi ('36) reported that "citrin" was not a pure substance but consisted of a mixture of hesperidin and eriodictyol glucoside. Bentsáth, Rusznyák and Szent-Györgyi ('37) have shown that hesperidin, or an impure sample of demethylated hesperidin, obtained from the mother-liquor of "citrin," possessed

"vitamin P" activity in scorbutic guinea pigs. They drew the general conclusion that the syndrome of experimental scurvy is due to a combined deficiency of vitamins C and P.

Zilva ('37a, '37b) repeated the work of the above authors and concluded that neither citrin, hesperidin, nor a mixture of hesperidin and eriodictyol, in doses of 1 mg, caused any delay in the onset of scurvy or the fatal termination of the disease in guinea pigs on a scorbutogenic diet. Moreover, post mortem examination of the animals confirmed this conclusion. The contradictory results obtained by Bentsáth, Rusznyák and Szent-Györgyi ('36, '37), and Zilva ('37a, '37b), have been explained by Zilva ('37b) and by Bentsáth and Szent-Györgyi ('37) as being due to the presence of traces of ascorbic acid, in amounts lower than the minimum prophylactic dose, in the scorbutogenic diet or the "citrin" used by the former workers, while the materials used by Zilva did not contain ascorbic acid. In a later article Szent-Györgyi ('38) reported that he was unable to reproduce his previous results and Moll ('38) came to the conclusion that hesperidin, among other things, did not effect the symptoms of guinea pigs on a vitamin C-free diet or on one containing a trace of the vitamin.

The present experimental work was undertaken to determine whether rutin, or its aglycone quercetin, would supplement or have a sparing action on subminimum doses of *l*-ascorbic acid in guinea pigs on a scorbutogenic diet.

#### GENERAL EXPERIMENTAL PROCEDURE

One hundred and seven young guinea pigs of both sexes ranging in weight from 209 to 425 gm (average, 318 gm) were divided equally, as nearly as possible, with reference to number, sex, and weight into 6 experimental groups in the following way: experiment 1, negative control group, consisted of 10 male and 9 female guinea pigs, of which 4 males and 4 females received daily 0.5 ml propylene glycol, as a control on the effect of propylene glycol; in experiment 2, 10 males and 8 females received rutin; in experiment 3, 10



males and 7 females received quercetin; in experiment 4, 9 males and 8 females received ascorbic acid; in experiment 5, 9 males and 7 females received both ascorbic acid and rutin; and in experiment 6, 8 males and 8 females received both ascorbic acid and quercetin. The animals were separated with reference to sex and kept in groups of three to 5 in screen-bottomed cages.

All guinea pigs were placed on the scorbutogenic diet described previously (Ambrose and DeEds, '47), but with the following modifications: the rolled oats were ground to about the same degree of fineness as the bran, and 3 gm of brewers' yeast and 1 gm of salt were added to each 100 gm of diet. Food and water were allowed ad libitum. One ml of U. S. P. cod liver oil was given to each animal by dropper every 5 days throughout the course of the experiment. All guinea pigs were weighed once each week. Measured volumes of all supplements were administered orally to each animal once each day. Supplements were prepared as follows: a 40% solution of rutin in propylene glycol and a 20% partial suspension of quercetin in propylene glycol were prepared fresh each week; ascorbic acid solutions were prepared fresh each day, just before administration, in glass-distilled water. Throughout the description of the experiments, "ascorbic acid" refers to *l*-ascorbic acid. Daily observations were made of all animals for symptoms of scurvy. At time of death the majority of the animals were X-rayed and post mortem examinations were made for bleeding gums or loosened teeth, hemorrhages into pleural and peritoneal cavities, hemorrhages in lungs, viscera and joints, and fragility of the long bones. Serum phosphatase determinations were made on some of the surviving guinea pigs at the close of the experiment by the method previously described (Ambrose and DeEds, '47). Of the 107 guinea pigs originally started with, only 4 animals were not considered in the evaluation of the results because they failed to withstand the rigors of the experiment for a minimum of 5 days.

## RESULTS

In experiment 1, in which approximately one-half of the guinea pigs received 0.5 ml each of propylene glycol daily until death ensued, no significant difference in survival time was noted between the sexes nor between those receiving propylene glycol and those not so treated. The onset and severity of the symptoms of scurvy were essentially the same for all animals in this group. The symptoms and post mortem changes described by Cohen and Mendel ('18) for scurvy in guinea pigs were typical of our findings: tenderness, swelling, stiffening of joints, and difficulty in the use of the hind legs. Hemorrhages into gums and loosening of teeth were not observed. Some tenderness and enlargement of the costochondral region were apparent in all of these animals. On autopsy, extensive hemorrhages into the joints and fragility of the bones were consistent findings. Occasionally some petechial hemorrhages were found in the subcutaneous tissues. The viscera of practically all animals appeared normal. In the lungs of the majority of the animals there was evidence of old and recent hemorrhages which we believe were due to lowered resistance to infection. X-ray pictures of 12 of the 19 animals were taken. In all cases there was marked evidence of changes in the rib and leg bones as compared with normal animals on a non-scorbutogenic diet. In the rib bones there appeared a thin white line along the periphery of the bone with increased porosity of the rest of the bone, findings which distinctly differentiate them from the rib bones of the positive control animals. The epiphyseal line of the leg bones showed slight decrease in calcium deposition along its course, accompanied by a decrease of calcium in both the epiphysis and diaphysis. All the leg bones appeared to show evidence of fracture in the metaphyseal region. No histological studies were made of these bones.

In the animals of experiment 2, receiving 100 mg rutin daily, no significant difference in survival of the two sexes was noted. The weight loss, onset of scorbutic symptoms,

post mortem findings of hemorrhages in the viscera and joints, and fragility of the bones were essentially the same as those observed in experiment 1. The X-ray pictures taken of 16 of the 18 animals showed the same results found in the previous group.

In experiment 3, quercetin (the aglycone of rutin) was administered to guinea pigs in order to compare its effect with that of rutin alone or in combination with subminimum amounts of ascorbic acid. Accordingly, the animals were given each day 50 mg of quercetin (approximate gram-molecular equivalent of 100 mg of rutin). The difference in survival time between the sexes was not statistically significant. The weight loss, onset of symptoms of scurvy, and findings at autopsy were the same as in experiment 1 and 2. Study of the X-ray pictures obtained on 5 males and 7 females confirmed the observations on the previous groups.

It has already been pointed out that the discrepancy between the results obtained by Bentsáth, Rusznyák and Szent-Györgyi ('36, '37) and those observed by Zilva ('37b) have been explained by the latter as being due possibly to the presence of small amounts of ascorbic acid under the experimental conditions of the former workers, whereas no ascorbic acid was present in the case of Zilva's work. The experiments described thus far in the present report have confirmed this explanation, since the longevity, picture of scury, and post mortem findings for the scorbutic controls and the scorbutic animals receiving rutin or quercetin without subminimum ascorbic acid supplements were essentially identical. The succeeding experiments are, therefore, concerned with a comparison of three groups of scorbutic guinea pigs receiving supplements of subminimum amounts of ascorbic acid, and this same supplement of ascorbic acid plus either rutin or quercetin, as used in experiments 2 and 3, to determine whether the flavonoids would have a sparing or synergistic action on the ascorbic acid. Accordingly, in the three experiments that follow, ascorbic acid was administered to the guinea pigs in doses of 0.2 mg/animal/day.

In experiment 4, in which the animals were given 0.2 mg of ascorbic acid each day, 10 of the animals died before termination of the experiment on the 44th day and the remaining 7 were sacrificed. The onset of symptoms of scurvy, contrary to the observations of Zilva ('37a, '37b), appeared at about the same time as for animals in the previous experiments. The weight loss of animals in this experiment did not exceed 10% each week, whereas for the negative controls (expt. 1) the weight loss was as much as 25% and for the animals receiving rutin (expt. 2) and quercetin (expt. 3) the loss was approximately 18%. Although all the animals showed evidence of swelling and stiffening of the joints, it was our impression from gross observation that this effect was not as great as in the previous experiments. On autopsy, recent bleeding into the joints was an uncommon finding. Out of 9 animals autopsied, one showed recent hemorrhage into the joints, two showed slight or no hemorrhages, and in 6 there was evidence of absorbed hemorrhages. Two of the latter also showed evidence of recent hemorrhage. The fragility of the bones of these animals was about the same as for the negative controls. X-ray examination of the long bones of these animals also showed essentially the same results observed in the case of the negative controls. Microscopical examination of longitudinal sections of the femur or tibia, stained with silver nitrate, showed distinct changes as compared with control animals on a non-scorbutogenic diet. Calcification along the epiphyseal line seemed to have increased, as did the porosity of the epiphysis and diaphysis. In other words, there appeared to have been a redistribution of calcium in these areas rather than a loss. In harmony with this interpretation is the fact that X-ray pictures revealed fracturing in the metaphyseal region.

In experiment 5, in which each animal received daily 0.2 mg ascorbic acid and 100 mg rutin, 7 of the animals died before termination of the experiment on the 45th day. The average weight loss was slightly less than that for animals in experiment 4, but this was not considered significant. How-

ever, the general appearance of these animals was much better than that of animals in any of the previous experiments. The symptoms so easily recognized in the other experiments were much less evident in the animals of this experiment. In only a few of the animals were swelling and stiffness of joints found. On autopsy, hemorrhages, either old or new, in the joints were found even less frequently than in experiment 4. X-ray studies of the long bones and microscopic examination of longitudinal sections of the femur or tibia revealed essentially the same findings as reported in connection with experiment 4.

In experiment 6, each animal received 50 mg of quercetin per day, the same amount used in experiment 3, in addition to 0.2 mg of ascorbic acid per animal per day. The per cent weight loss of these animals, general appearance, symptoms of scurvy, and findings at autopsy were about the same as in experiment 5. The results of X-ray studies and microscopic examination of sections of femur or tibia did not differ from those of experiment 4.

#### *Serum phosphatase studies*

It has been concluded by Robison ('32) and confirmed by Gould and Shwachman ('43) that serum phosphatase activity in guinea pigs is of osteoblastic origin, and that in scurvy there is an impairment of osteoblastic activity with a concomitant rise or fall in blood serum phosphatase activity, depending on whether or not ascorbic acid supplements are administered in conjunction with the scorbutogenic diet. The present serum phosphatase studies were, therefore, made to determine whether or not such determinations might serve to demonstrate objectively that rutin or quercetin had a synergistic or "sparing action" on the small supplements of ascorbic acid used.

Serum phosphatase determinations were not made at the beginning nor during the course of the various experiments because of the large amount of blood required and the danger of loss of the animals. Therefore, determinations were

made only on animals surviving at the termination of experiments 4, 5 and 6 on the 44th; 45th and 49th days, respectively.

Serum phosphatase determinations were made on 6 of the surviving guinea pigs in experiment 4, on 8 in experiment 5, and on three of the 4 survivors in experiment 6. The values obtained, with their range, are as follows: 6.6 (5.3–8.6); 6.5 (3.4–10); and 7.0 (5.0–9.8), respectively.

As judged by our previous studies (Ambrose and DeEds, '47) and the data reported by Gould and Shwachman ('43), the values for phosphatase activity found are about one-half those for normal guinea pigs. Under the conditions of our experiments and those of Gould and Shwachman, the dose of ascorbic acid (0.2 mg per day) would tend to hold the serum phosphatase values at the level found. In other words, the administration of rutin or quercetin had no significant influence on the phosphatase activity, and the observed increase in longevity must be explained in some other way. In earlier studies (unpublished data) serum phosphatase values continued to fall in guinea pigs on a scorbutogenic diet receiving a daily supplement of 100 mg of rutin without added ascorbic acid.

### *Effects on longevity*

The determination of serum phosphatase activity in experiments 4, 5 and 6 necessitated termination of the experiments before the survival time of all the animals had been determined. The median survival time of all the animals in experiments 1, 2 and 3 cannot be compared fairly with the median survival time of the weakest or more susceptible animals which died before termination of the last three experiments. However, it would be fair to compare the median survival time of the first 7 animals dying in each experimental group, since the groups involved approximately the same numbers of guinea pigs at the beginning of the experiments. The lowest mortality occurred in experiment 5, when the scorbutogenic diet was supplemented with a

subminimum amount of ascorbic acid plus rutin. A comparison has, therefore, been made of the median survival time of these 7 animals with that of the first 7 animals dying in each of the other experimental groups. The median survival time in the first three experiments was 12 to 15 days. In experimental groups 4 and 6, receiving ascorbic acid and ascorbic acid and quercetin, the median survival times were 20 and 22 days, respectively. In experimental group 5, receiving ascorbic acid and rutin, the median survival time was 30 days. From these data it appears that ascorbic acid and rutin, when administered simultaneously, prolonged the life of guinea pigs beyond that of animals receiving ascorbic acid, rutin, or quercetin separately, under the conditions of our experiments.

#### DISCUSSION

The results of the above experiments are in harmony with the conclusion of Bentsáth, Ruzsnyák and Szent-Györgyi ('37) that the syndrome of experimental scurvy is due to the combined deficiency of vitamins C and P. However, guinea pigs on a scorbutogenic diet without the addition of subminimum amounts of ascorbic acid (i.e., amounts less than the minimum prophylactic dose), but receiving rutin or quercetin, develop symptoms of scurvy at about the same time as guinea pigs receiving no supplements of rutin or quercetin. This is in agreement with the results of Zilva ('37a, '37b), who used citrin, hesperidin, and a mixture of hesperidin and eriodictyol, but is not entirely in accord with the claim of Bentsáth and co-workers ('36) that animals receiving citrin alone showed fewer hemorrhages than the negative controls.

When subminimum amounts of ascorbic acid were fed to guinea pigs on a scorbutogenic diet, the symptoms of scurvy were definitely less severe than the symptoms of scorbutic guinea pigs not receiving such supplements. When the scorbutogenic diet was supplemented with rutin or quercetin in addition to the subminimum dose of ascorbic acid, the symp-

toms of scurvy remained about the same as in guinea pigs receiving the ascorbic acid alone, except that bleeding into the joints was a less common finding. This observation confirms the work of Bentsáth and co-workers ('36) if we accept Zilva's explanation that traces of ascorbic acid were present under the existing experimental conditions.

Neither rutin nor quercetin fed as a supplement to guinea pigs on a scorbutogenic diet had any effect on longevity. When rutin was administered together with subminimum

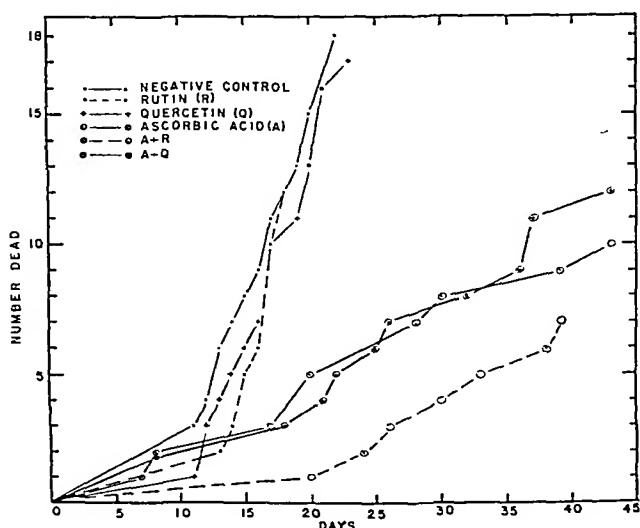


Fig. 1 The comparative effect of rutin (R), quercetin (Q), ascorbic acid (A) and mixtures of ascorbic acid and rutin (A+R), and ascorbic acid and quercetin (A+Q), on longevity in guinea pigs on a scorbutogenic diet.

doses of ascorbic acid, life was definitely prolonged. This is shown in figure 1, where it can readily be seen that rutin appears to be more effective than an equi-molecular amount of quercetin. This difference is believed to be due to the poorer solubility of pure quercetin in propylene glycol and body fluids. In preliminary studies (unpublished data) a less pure sample of quercetin extracted from goldenrod was used. This sample was more soluble in propylene glycol and the data obtained indicated that it was equally as good as



rutin, on an equi-molecular basis, in prolonging life. It is quite unlikely that the impurities of this sample contained ascorbic acid because of the extraction process used. Figure 1 shows that the first guinea pig on a scorbutogenic diet plus supplements of ascorbic acid and rutin died on the 20th day of the experiment. By this time all but three of the negative controls, 5 of the animals receiving ascorbic acid without rutin, and all but three receiving only rutin supplements and 4 receiving only quercetin supplements had died.

Since completion of this investigation a report by Cotereau, Gabe, Gero and Parrot ('48) has appeared in which the authors claim a greater storage of ascorbic acid in the liver, spleen, kidneys and adrenals of guinea pigs on a scorbutogenic diet receiving daily supplements of ascorbic acid and catechin (a mixture of epimers of d-catechin—Perrcapyl Russell) than in guinea pigs receiving ascorbic acid or catechin alone. At autopsy the scorbutic type of lesions was found in all the animals except those given ascorbic acid and catechin.

In the experiments presently reported the scorbutic lesions were less pronounced in animals receiving ascorbic acid with supplements of rutin or quercetin than in animals receiving ascorbic acid alone. However, the lesions in the animals receiving ascorbic acid alone were less severe than in the negative controls, and also less severe than in the guinea pigs receiving rutin or quercetin without ascorbic acid.

Cotereau, Gabe, Gero and Parrot ('48) concluded that storage of ascorbic acid occurs in the presence of a "sparing factor" such as catechin, and this *in vivo* protection of ascorbic acid is consistent with the observation of Parrot and Cotereau ('46) that catechin retards the rate of oxidation of ascorbic acid *in vitro*. Similarly, the present report on the value of rutin and quercetin in supplementing sub-minimum amounts of ascorbic acid in scorbutic guinea pigs is in agreement with unpublished data obtained in this laboratory on the antioxidant action of these flavonoids toward ascorbic acid.

## SUMMARY AND CONCLUSIONS

Data are presented on the effects of rutin and quercetin in guinea pigs maintained on a scorbutogenic diet with and without the addition of subminimum amounts of ascorbic acid.

Rutin and quercetin administered orally in doses of 100 and 50 mg per animal per day, respectively, did not prolong the life of the guinea pigs beyond that of the negative control animals on the scorbutogenic diet alone. The scorbutic symptoms and the post mortem findings did not differ from those of the negative controls.

In guinea pigs receiving subminimum amounts of ascorbic acid alone, ascorbic acid and rutin, and ascorbic acid in combination with quercetin, the post mortem findings were essentially the same except for the fact that the last two groups showed fewer fresh hemorrhages than the animals receiving ascorbic acid alone. Microscopic study of stained longitudinal sections of the femurs or tibias of these three groups of animals showed essentially the same picture for all groups. This picture differed from that of positive control animals on a non-scorbutogenic diet in that the epiphyseal line showed slightly more calcification and the epiphysis and diaphysis greater porosity, which might account for the fractures in the metaphyseal region seen in X-ray pictures. The combined supplements of ascorbic acid and rutin apparently prolonged the life of scorbutic guinea pigs.

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# THE EFFECT OF RETENTION OF NITROGEN IN CASEIN OR LACTALBUMIN HYDROLYSATES ON THE REGENERATION OF PLASMA PROTEINS OF PROTEIN- DEPLETED DOGS

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In previous communications (Chow, '46; Bolling, Block and Chow, '47; Chow, Seeley, Allison and Cole, '48) data were presented to demonstrate that oral administration of lactalbumin or its hydrolysate to protein-depleted dogs favors the regeneration of plasma albumin, whereas casein or its hydrolysate stimulates the production of both albumin and globulins. Since the chemical analyses of the 10 essential and a few of the non-essential amino acids showed a marked similarity in the two hydrolysates, the difference in the plasma protein regeneration properties is believed not to be due (Bolling, Block and Chow, '47) to the difference in amino acid composition alone. Hence, an explanation is yet to be found.

The superiority of lactalbumin over casein in promoting the growth of young rats (Supplee and Clark, '46) or in supporting nitrogen balance in dogs (Allison, '48) has been well demonstrated, and the difference can be compensated for by feeding the animals a higher level of casein. It is,

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therefore, of interest to ascertain whether the production of specific protein components in plasma is similarly dependent on the amount of absorbed nitrogen, which might then account for the difference in the plasma protein regeneration properties of these two milk proteins. To this end studies were undertaken which involved feeding a group of protein-depleted dogs with a casein hydrolysate at a nitrogen level which would stimulate a rapid and pronounced regeneration of plasma proteins. For comparison, three other groups of protein-depleted animals were fed three different levels of a tryptic digest of lactalbumin, so that the retained nitrogen was either greater, approximately equal to, or less than that retained by dogs receiving the casein hydrolysate. In addition, two other groups of dogs were fed the same level of casein hydrolysate as the first group but were given 1 or 2% of methionine as a supplement in order to increase the nitrogen retention (Allison, Anderson and Seeley, '47; Brush, Willman and Swanson, '47; Cox, Mueller, Elman, Albanese, Kemmerer, Barton and Holt, '47) to approximately that of the animals receiving the highest dosage of lactalbumin hydrolysate. The regeneration of various protein components in the plasma of the dogs fed these diets was measured by determining the composition of plasma electrophoretically, and the total circulating plasma proteins (the product of the plasma protein concentration and the plasma volume). The nitrogen retention was also measured for the whole period of feeding. The results are reported in the present paper.

## EXPERIMENTAL

### *Preparation of hydrolysates*

One kg of edible casein<sup>2</sup> was suspended in 9 l of distilled water previously warmed to 50°C. Enough 6 N NaOH (usually 85 to 90 ml) was added with continuous stirring until

<sup>2</sup> Borden's.

all the protein was in an even suspension and the pH remained about 7.6 for at least 30 minutes. Forty grams of a purified trypsin preparation (1:280)<sup>3</sup> were added and the suspension incubated at about 50°C. for 38 hours, after which time 900 gm of Celite no. 505 were added to aid filtration. The digestion was considered complete when the addition of an equal volume of a 5% trichloroacetic acid solution to a small aliquot of the filtrate did not produce any opalescence. The water-clear filtrate was lyophilized. Chemical analysis showed that such hydrolysates contained about 2.0% sodium and 13.5% total nitrogen, of which 28 to 30% was liberated as amino nitrogen, as determined by the nitrous acid method.

Similarly, lactalbumin hydrolysate was prepared by suspending 1 kg of lactalbumin<sup>4</sup> in 9 l of water kept at 37°C. The pH was kept between 7 and 8 by continuous addition of 6 N NaOH. Forty grams of the purified pancreatic enzymes were added. During the first 6 to 8 hours the digestion took place rapidly, and the original pH was maintained by further addition of alkali. Incubation at 37°C. was allowed to proceed for 48 to 72 hours. The suspension was then adjusted to pH 5 by the addition of a 4 N HCl solution and filtered with the aid of Celite (100 gm/liter), and the filtrate was lyophilized. Chemical analysis showed that the hydrolysate contained 2.5% sodium and 12.8% nitrogen, of which 35 to 40% was liberated as amino nitrogen.

### *Preparation of hypoproteinemic dogs*

Normal and healthy dogs were given a protein-free diet consisting of 21% sucrose, 19.4% dextrose, 32.9% dextrin, 21.8% lard, 1.8% Osborne-Mendel salt mixture, and 2.5% agar, with the following vitamins as supplements: thiamine, riboflavin, nicotinic acid, calcium pantothenate, pyridoxine, choline-2-methyl-1-naphthoquinone, biotin and vitamins A and D. These dogs were given daily an amount of this diet

<sup>3</sup> Obtained from the Wilson Laboratories.

<sup>4</sup> Borden 15-42.

calculated to supply 80 cal. per kilogram body weight. After a period of 6 to 8 weeks the plasma protein concentration dropped to 4.5 to 5.2 gm % and the albumin-to-globulin ratio was reduced to approximately 0.25.

### *Repletion with different test proteins*

The protein-depleted dogs were repleted with the test diets also at 80 cal. per kilogram body weight per day at different nitrogen levels. Determinations of plasma volume (Gregersen and Stewart, '39) and the plasma protein concentrations, as well as electrophoretic analyses of plasma (Longsworth, '42) were performed before and after depletion, and also after three or 6 weeks of repletion with the test substances. It was thus possible to study quantitatively the changes of each plasma protein component during the course of protein administration. The technique of these determinations has been published elsewhere (Chow, Seeley, Allison and Cole, '48) and need not be repeated.

### RESULTS

Six groups of protein-depleted dogs were fed for 6 weeks three levels of lactalbumin hydrolysate (0.35, 0.25 and 0.15 gm N/kg body weight per day) and one level of casein hydrolysate (0.35 gm N), with or without the supplementation of 1 or 2% methionine. In the 7th week the animals were given a protein-free diet. The nitrogen intake as well as excretion for the 7 weeks were determined on the basis of a weekly collection. The biological value, or the fraction of the hydrolysate nitrogen retained in the body of the test animals, was calculated according to the equation of Allison ('46):  $(NB) = (BV)(AN) - (EN)$ , where  $(NB)$  = nitrogen balance,  $(BV)$  = biological value,  $(AN)$  = absorbed nitrogen and  $(EN)$  = endogenous nitrogen. It was found the digestibility of the hydrolysate diet was essentially complete. Hence, the  $(AN)$  value approached closely that for nitrogen intake  $(IN)$ .



The amount of plasma protein regeneration was determined by comparing the total circulating plasma proteins before and after a given period of repletion.

TABLE 1

*The levels of absorbed nitrogen and the plasma protein regeneration*

PROTEIN SUPPLEMENT	NO. DOGS USED	LEVEL OF INTAKE <sup>1</sup>	NITROGEN BALANCE <sup>2</sup>	RETAINED "BIOLOGICAL VALUE" <sup>3</sup>	PLASMA PROTEIN NITROGEN <sup>4</sup>
					%
Lactalbumin hydrolysate	5	0.35	+ 0.186	0.8	4.6
Lactalbumin hydrolysate	4	0.25	+ 0.118	0.8	3.0
Lactalbumin hydrolysate	4	0.15	+ 0.072	1.0	2.7
Casein hydrolysate	5	0.35	+ 0.117	0.5	4.5
Casein hydrolysate + 1% methionine	3	0.35	+ 0.175	0.7	4.5
Casein hydrolysate + 2% methionine	3	0.35	+ 0.165	0.7	5.0

<sup>1</sup> Level of intake is expressed as grams nitrogen per kilogram body weight per day.

<sup>2</sup> Nitrogen balance is the difference between nitrogen intake and nitrogen excretion, expressed in grams nitrogen per kilogram body weight per day.

<sup>3</sup> "Biological value" is calculated according to the equation of Allison ('46):  $(NB = (BV) (AN) - (EN))$ . (See text.)

<sup>4</sup> Per cent of the total absorbed nitrogen used for the synthesis of total plasma protein.

### *The relationship between absorbed nitrogen and plasma protein synthesis*

The results given in table 1 demonstrate that all 6 diets were adequate in supporting nitrogen balance (see column 4) during the period of repletion. At the level of nitrogen intake of 0.35 gm/kg body weight per day, the lactalbumin hydrolysate was considerably more effective than the casein hydrolysate, which was improved by the addition of 1 or 2% methionine. As was to be expected, the extent of nitrogen balance was dependent on the dosages of lactalbumin hydrolysate given, being highest at 0.35 gm N intake.

The results given in column 5 of table 1 demonstrate that half of the casein hydrolysate nitrogen<sup>5</sup> was retained, and the retention was increased to 70% by the addition of 1 or 2% methionine. On the other hand, at least 80% of the lactalbumin hydrolysate nitrogen was retained; the retention was virtually complete when the level of nitrogen administered was reduced. The results shown in this table also demonstrate that, of the total retained nitrogen, approximately 5% was used for the synthesis of plasma protein (see column 6). This percentage was decreased to about three as the nitrogen retention was lowered.

### *The synthesis of plasma albumin and globulins*

The difference in the plasma protein regeneration patterns of a series of dogs fed with lactalbumin hydrolysate or with casein hydrolysate is further illustrated in table 2. The data (column 3) demonstrate that after 6 weeks of repletion dogs fed with lactalbumin hydrolysate regenerated less plasma proteins than those fed with casein hydrolysate. However, the animals in the former group showed striking increase in the albumin and essentially no increase in globulins, whereas animals fed with casein hydrolysate showed a marked increase in both the albumin and globulin fractions after 6 weeks of feeding. Supplementation with methionine for the same period resulted in a definite increase (though less marked) in the amount of globulin regenerated. The changes in A/G ratio, recorded in the last two columns of table 2, demonstrate that this ratio was the highest (0.60) for the group of dogs fed with lactalbumin hydrolysate at

<sup>5</sup> The biological values of the casein and lactalbumin hydrolysates obtained under our experimental conditions were considerably lower than those reported by Allison ('46). However, it must be pointed out that our data represent the average values after 6 weeks of feeding, during which time the state of protein reserves of the animals was gradually changing from that of deficiency to that of abundance. In Allison's experiments the determination was performed over a much shorter period and at such a dosage that the protein reserves of the dogs did not materially change.

TABLE 2  
*The effect of oral feeding of casein or lactalbumin hydrolysates at different levels of nitrogen intake on total circulating plasma proteins of dogs depleted in proteins*

LEVEL OF FEEDING	NO. DOGS	TOTAL CIRCULATING PROTEINS <sup>1</sup>		TOTAL CIRCULATING ALBUMIN <sup>1</sup>		TOTAL CIRCULATING GLOBULINS <sup>1</sup>		A/G
		3 weeks	6 weeks	3 weeks	6 weeks	3 weeks	6 weeks	
Lactalbumin hydrolysate: 0.35 gm N/kg	4	115 ± 10.0	128 ± 10.0	185 ± 21	252 ± 23	100 ± 8.6	100 ± 9.3	0.22 0.60
Lactalbumin hydrolysate: 0.25 gm N/kg	4	121 ± 7.1	137 ± 8.7	193 ± 21	251 ± 40	106 ± 5.2	105 ± 6.5	0.23 0.46
Lactalbumin hydrolysate: 0.15 gm N/kg	4	108 ± 9.3	123 ± 7.1	176 ± 22	227 ± 16	97 ± 9.6	105 ± 7.0	0.16 0.35
Casein hydrolysate: (0.35 gm N/kg) plus 1% methionine	3	124 ± 6.1	141 ± 9.0	172 ± 23	245 ± 35	114 ± 5.7	120 ± 3.0	0.23 0.41
Casein hydrolysate: (0.35 gm N/kg) plus 2% methionine	3	133 ± 7.9	146 ± 6.1	196 ± 18	262 ± 29	116 ± 4.8	118 ± 1.0	0.26 0.44
Casein hydrolysate: (0.35 gm N/kg) alone	5	138 ± 7.1	150 ± 2.8	195 ± 7.5	210 ± 10	113 ± 10.6	136 ± 9.1	0.22 0.51

<sup>1</sup> Calculated as per cent of the amount determined prior to protein feeding, with standard error.

TABLE 3

*The effect of oral feeding of casein or lactalbumin hydrolysates at different levels of nitrogen intake on total circulating globulins in the plasma of dogs depleted in proteins*

LEVEL OF FEEDING	DOGS NO.	TOTAL CIRCULATING ALPHA (1 AND 2) <sup>1</sup>		TOTAL CIRCULATING GAMMA <sup>1</sup>		TOTAL CIRCULATING OTHER GLOBULINS <sup>1</sup>	
		3 weeks	6 weeks	3 weeks	6 weeks	3 weeks	6 weeks
0.35 Lactalbumin hydrolysate	4	78 ± 11.2	63 ± 5.2	73 ± 8.7	96 ± 14.3	113 ± 9.6	115 ± 16.2
0.25 Lactalbumin hydrolysate	4	64 ± 5.2	56 ± 9.6	98 ± 6.7	114 ± 10.1	135 ± 6.1	152 ± 6.3
0.15 Lactalbumin hydrolysate	4	71 ± 11.8	83 ± 15.4	81 ± 5.7	114 ± 7.2	117 ± 10.8	133 ± 4.9
Casein hydrolysate plus 1% methionine	3	90 ± 13.7	88 ± 5.4	129 ± 6.0	141 ± 13.3	135 ± 8.3	130 ± 14.7
Casein hydrolysate plus 2% methionine	3	136 ± 9.1	143 ± 15.0	93 ± 5.7	93 ± 8.3	129 ± 9.0	137 ± 9.4
Casein hydrolysate alone	5	128.7 ± 11.4	133.6 ± 12.9	106 ± 16.7	118.6 ± 20.0	130.3 ± 14.2	142.4 ± 12.2

<sup>1</sup> Calculated as per cent of the amount determined prior to protein feeding, with standard error.

a level of 0.35 gm N. The ratio of this group of animals approached unity when the repletion was continued for 10 weeks. On the other hand, repletion of dogs with casein hydrolysate for the same length of time did not significantly increase this ratio.

The effect of repletion with different dietary proteins on the globulin fractions was also investigated. The results (see table 3) demonstrate that the oral administration of lactalbumin hydrolysate at any one of three levels caused a marked drop in the total circulating alpha globulins. In contrast to this, the feeding of casein hydrolysate, with or without methionine supplementation, to the protein-depleted animals brought about no marked decrease and, as matter of fact, an increase of these globulins. In two cases no sharp difference in the effect of feeding these two hydrolysates on the regeneration of the gamma globulin was observed. Either hydrolysate brought about a marked increase in "other globulins," i.e., the total globulins minus the gamma and alpha globulins.

#### DISCUSSION

The classic studies of Rose and his associates on the physiological and nutritional properties of amino acids demonstrate the possibility of obtaining essentially normal growth in young rats by feeding the protein elements of the diet in the form of 10 pure amino acids. The removal of any one of these particular amino acids resulted in impaired growth, or in considerable losses in weight and ultimately death. More recent investigations (Wooley, '47; Womack and Rose, '46) demonstrate that in addition to the 10 essential amino acids, the presence of some unknown nutritive factors in the diet is essential for normal growth. The destruction of these factors in crude casein by acid hydrolysis or their removal by extraction with organic solvents results in sub-optimum growth in rats. Relatively little attention has been directed to the possible role of these yet unidentified nu-

tritional factors which may predetermine the synthesis of a particular type of plasma protein component.

Since the difference in the plasma protein regeneration properties of casein and lactalbumin is due neither to the amino acid composition nor to the amount of the amino acid retained by the animals, it is not unlikely that nutritional factors other than the essential amino acids play a role in directing the synthesis of plasma proteins. Thus it is no longer safe to assume that the mere presence of the essential amino acids, even in great abundance, and of all the known vitamins can assure us of optimum nutrition, either for maintenance or for convalescence.

#### SUMMARY

Protein-depleted dogs were fed one level of casein hydrolysate. For comparison, three other groups of dogs were given different levels of lactalbumin hydrolysate so that the retained nitrogen was either greater, approximately equal to, or less than that in dogs receiving the casein hydrolysate. In addition, two other groups of dogs were administered the casein hydrolysate orally with the supplementation of 1 or 2% methionine, in order to increase the nitrogen retention to approximately that retained by the animals receiving the highest dosage of lactalbumin hydrolysate. The total nitrogen absorbed and the regeneration of various plasma protein components were determined quantitatively. The data demonstrated that the lactalbumin hydrolysate given at any one of the levels stimulated the production of albumin and led to a marked decrease in alpha globulins. On the other hand, casein hydrolysate favored the production of albumin and globulins even on supplementation with methionine, which increased the absorption of the casein hydrolysate nitrogen fed. The results, therefore, led to the conclusion that the difference in the plasma protein regeneration properties of the two protein hydrolysates is not due to the difference in the amount of absorbed nitrogen, but perhaps to the existence of an unidentified nutritional factor.

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# THE ENHANCEMENT OF THE NUTRITIVE VALUE OF WHEAT GLUTEN BY SUPPLEMENTATION WITH LYSINE, AS DETERMINED FROM NITROGEN BALANCE INDICES IN HUMAN SUBJECTS <sup>1,2</sup>

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ONE FIGURE

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In a recent paper from this laboratory (Hoffman et al., '48) it was demonstrated that the method of Allison and Anderson ('45) for the determination of the so-called nitrogen balance index in dogs with induced hypoproteinemia could be applied to human hospital patients with protein deficiency. In that report the nitrogen balance index of a lyophilized amino acid preparation administered intravenously was found to be relatively constant in 20 subjects in spite of marked variability in their requirements of nitrogen for equilibrium. The latter values were shown to be dependent upon the nitrogen excretion on a protein free diet. This excretion varied greatly, being influenced by the degree and duration of protein deficiency, by the nature of the illness producing this deficiency, and by unknown factors. It was believed that the nitrogen balance index was a reliable gauge of the biological value of protein for human protein-deficient subjects.

<sup>1</sup>These studies were aided by a grant from the Interchemical Corporation, Biochemical Division, Union, New Jersey.

<sup>2</sup>Presented before the Federation of American Societies for Experimental Biology, March 18, 1948.

As a further extension of the Allison method, a comparison has been made of the nitrogen balance index of a specially processed gluten preparation with that of the same product reinforced with 4% lysine. The studies have been carried out on 10 hospital patients, of whom 7 had recognizable protein deficiency and three had serum protein concentrations within normal limits but might have had mild protein deficiency.

#### EXPERIMENTAL

As in previous nutritional investigations made in this laboratory, the nitrogen balance studies were executed in a special metabolic unit on a surgical ward of the hospital; the unit was staffed 24 hours a day by nurses specially chosen for this function. The urine specimens were collected in bottles containing toluene for each 24-hour period and were analyzed immediately. Stools were collected in covered enamel jars, in which they were prepared for nitrogen analysis by homogenization with water and concentrated sulfuric acid, as suggested by Peters and Van Slyke ('32). Nitrogen determinations of the urine, stool and blood specimens were made by the photometric micro-Kjeldahl method described by Hoffman and Osgood ('40). These were frequently checked by the macro-Kjeldahl method utilized for the nitrogen analysis of the food samples. In the latter method, digestion was accomplished with sulfuric acid, potassium sulfate, and mercuric oxide. Sodium hydroxide with sodium sulfide were employed for the liberation of ammonia, which was distilled into a boric acid solution and titrated. As a check for completeness of the urine collection, daily creatinine determinations of the 24-hour urine specimen were made.

The basic non-protein diet consisted of a high carbohydrate drink,<sup>3</sup> soup made of carbohydrate, vegetable oil, and a nitrogen-poor vegetable; and a specially made protein-free candy.

<sup>3</sup> Made up of Cartose, provided by H. W. Kinney and Sons, Columbus, Indiana, and a beverage base.

The gluten preparations, with and without added lysine,<sup>4</sup> were incorporated in the diet in the form of muffins or puddings. Salt was allowed *ad libitum* in the soup, and was added in the preparation of the muffins and puddings. Vitamins were provided in the form of a commercial mixture,<sup>5</sup> 1 ml daily intramuscularly, and ascorbic acid, 150 mg daily by mouth. The total caloric intake was maintained at 35 to 40 cal. per kilogram per day. The average nitrogen content of the basic daily diet was found to be 400 mg. This quantity was not included in the calculation of nitrogen balance, since it was from poor protein sources, was constant through all the periods, and was probably of the same magnitude as the undeterminable nitrogen excretions. At worst, the error involved was small and did not affect the determination of the nitrogen balance index.

Two groups of nitrogen balance experiments were performed. In the first group of three subjects the balance studies were carried out as in the previously reported series, with progressive increases in the nitrogen intake after the nitrogen output on the basic diet alone had been determined. (In all 10 experiments an adjustment period of two days on the basic diet was allowed before specimens were collected, since it had been previously found that the nitrogen excretion in these first days was too high to be truly representative of the protein-free period.) After 5 days on gluten, the subjects were given the same quantity of gluten reinforced with 4% L-lysine for a period of 5 days. Then a large quantity of gluten was administered for another 5-day period, followed by 5 days of an isonitrogenous quantity of gluten plus lysine.

<sup>4</sup> These were kindly furnished by the Interchemical Corporation, Biochemical Division, Union, New Jersey. The gluten was a specially prepared powder of non-agenized wheat gluten. The *gluten-plus-lysine* was also a specially prepared powder, consisting of non-agenized wheat gluten homogeneously mixed with 4% purified L-lysine hydrochloride. This latter product is now being marketed as Elutein.

<sup>5</sup> Betalin Complex, kindly furnished by Eli Lilly and Company, Indianapolis, Indiana.

This alternation was continued until good positive balances were obtained with both products.

In the second group of 7 subjects the nitrogen balance experiments were carried out with only two points for the determination of the curve, on the assumption that the straight line curves demonstrated in the first three subjects would apply equally well here. In these studies, after a variable protein-free period ranging from three to 10 days, the subjects received for 8 to 10 days a quantity of gluten calculated to give a slightly negative or positive nitrogen balance. This diet then was replaced by an isonitrogenous diet of gluten plus lysine for another 10-day period. In three of the experiments it was possible to return the patients to the original gluten diet for another 10-day period.

#### RESULTS

The nitrogen balance findings in the first group of three subjects are shown in table 1. When the nitrogen balance is plotted against the nitrogen intake (see fig. 1), the points fall on a straight line curve both for gluten and for gluten plus lysine, as predicted by Allison. The Allison equation is  $NB = K(AN) - NE_o$ , where  $NB$  is the nitrogen balance,  $AN$  is the absorbed nitrogen,  $NE_o$  is the nitrogen excretion on the protein-free diet, and  $K$  is the slope of the curve or the nitrogen balance index. The nearer  $K$  approached the value of one, the higher was the biological value of the protein studied. It is possible to substitute  $NI$ , the nitrogen intake, for  $AN$ , the absorbed nitrogen, in these experiments, for the fecal nitrogen excretion was not found to be any higher during the protein feedings than during the days of the protein-free diet.

The  $K$  values for the gluten curves, which can be calculated either as  $(NB + NE_o)/AN$  or as  $NE_o/EN$  where  $EN$  is the nitrogen requirement for nitrogen equilibrium, were 0.53, 0.53 and 0.59, respectively, for subjects 1, 2 and 3. The  $K$  value for gluten plus lysine was significantly higher in each of the three experiments, being 0.60, 0.67 and 0.69, respec-

TABLE 1  
*Nitrogen balances and nitrogen balance indices (K) in patients on diets of gradually increased quantities of gluten (G)*  
*or gluten plus lysine (GL)*

SUBJECT	DIET PERIOD	NITROGEN INTAKE			NITROGEN EXCRETION			NITROGEN BALANCE		K	LN <sup>1</sup>
		gm/day	mg/kg/day	days	gm/day	mg/kg/day	gm/day	mg/kg/day	mg/kg		
1 M.M., 68 yrs. Male, 68 yrs. leg ulcer Wt., 69.5 kg Tot. protein 6.59 gm/100 ml	Control	0	0	3	3.49	50.2	—	—	—		
	G	4.52	65.1	5	5.59	80.4	—	—	—		
	GL	4.60	66.0	5	5.32	76.5	—	—	—		
	G	6.79	97.5	5	6.34	91.0	—	—	—		
	GL	6.90	99.2	5	5.80	83.2	—	—	—		
	G	9.05	130.0	5	7.30	105.0	—	—	—		
	GL	9.20	132.0	5	6.95	100.0	—	—	—		
2 L.L., Female, 68 yrs. Garcinoma of uterus Wt., 43.5 kg Tot. protein 4.57 gm/100 ml	Control	0	0	3	1.86	42.8	—	—	—		
	G	2.15	49.4	5	2.88	66.2	—	—	—		
	GL	2.05	47.1	5	2.60	58.8	—	—	—		
	G	4.30	98.8	5	3.91	89.8	—	—	—		
	GL	4.10	94.2	5	3.27	75.2	—	—	—		
	G	9.30	213.8	5	7.49	172.2	—	—	—		
	GL	9.02	207.3	5	6.73	154.7	—	—	—		
3 N.D., Female, 60 yrs. Amputated leg Wt., 30.0 kg Tot. protein 6.12 gm/100 ml	Control	0	0	4	1.80	60.0	—	—	—		
	G	2.15	71.7	5	2.68	89.3	—	—	—		
	GL	2.05	68.3	5	2.44	81.3	—	—	—		
	G	1.30	143.3	5	3.57	119.0	—	—	—		
	GL	4.10	137.0	5	3.18	106.1	—	—	—		
	G	9.30	310.0	5	6.58	219.5	—	—	—		
	GL	9.02	301.3	5	6.28	209.4	—	—	—		

<sup>1</sup> Nitrogen requirement for nitrogen equilibrium.

tively. Correspondingly, the nitrogen requirement for nitrogen equilibrium (EN), which is the point where the curve crosses the zero nitrogen balance line, was 94, 81 and 102 mg per kilogram per day, respectively, for gluten. It was significantly lower in each case for gluten plus lysine, namely, 83, 64 and 87 mg per kilogram per day, respectively.

Table 2 gives the data obtained in the 7 experiments in the second group. The K values were calculated from the straight lines drawn from the point of zero nitrogen intake through the single point of gluten or gluten plus lysine feeding.

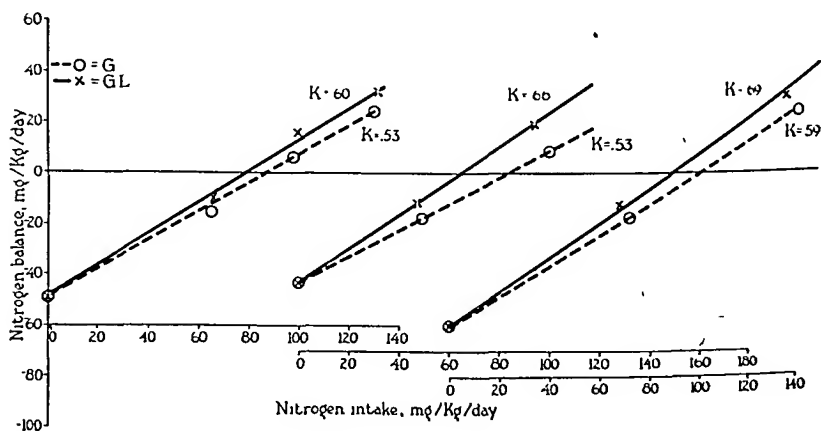


Fig. 1 Curves of nitrogen balance in relation to intake in subjects 1, 2 and 3. K in each case is the slope of the straight line curve and is the nitrogen balance index.

The nitrogen requirement for equilibrium was estimated by extension of the straight line curve through the line of zero nitrogen balance. In 6 of the 7 subjects, the K values for gluten ranged from 0.53 to 0.78, while those for gluten plus lysine ranged from 0.76 to 0.87. These values were of the same order as those in the three experiments of the first group, which indicates that no great error was introduced in drawing the rectilinear curves from only two points. In each experiment the K value for gluten plus lysine was higher than that of gluten. In the case of subject 7, the K value for gluten was 0.80 and for gluten plus lysine 0.98. Though

the values found in this case were consistent with the others in that the K for gluten plus lysine was higher than that for gluten, the absolute values were improbably high. The deviation from the mean of the K values in this case was found to be more than twice the standard deviation for both gluten and gluten plus lysine. For this reason, the values in this experiment were not utilized in the final statistical evaluation.

In subjects 4, 8 and 9, a second period of 10 days on the original quantity of gluten produced K values not appreciably different from those of the initial gluten periods. Even in the two instances in which the K value was improved in the second gluten period, this value was significantly lower than that for gluten plus lysine in the same subject. For the statistical evaluation the K and EN values for gluten in these subjects were calculated from the mean of the values from the two gluten periods.

For the total of 9 experiments in both groups the mean K value for gluten was 0.62, with a standard deviation of  $\pm 0.09$  (14.5%), and for gluten plus lysine it was 0.76, with a standard deviation of  $\pm 0.08$  (10.5%). The difference between these two means was significant, the  $t$  value<sup>6</sup> for statistical significance being 3.5. Similarly, for the total of 9 experiments, the mean EN for gluten was 80 mg per kilogram per day, with a standard deviation of  $\pm 14$  mg (17.5%), while that for gluten plus lysine was 65 mg per kilogram per day, with a standard deviation of  $\pm 11$  mg (17%). Again the difference between the means was significant, the  $t$  value being 2.6.

<sup>6</sup>The  $t$  value of the statistically significant difference between the average of two sets of experimental values was determined essentially by the method of Snedecor ('46), as follows:

$$t = \frac{a_1 - a_2}{\sqrt{\frac{\sigma_1^2}{n_1} + \frac{\sigma_2^2}{n_2}}}$$

where  $a_1$ ,  $\sigma_1$ , and  $n_1$  are, respectively, the mean, the standard deviation, and the number of experiments in the first group, and  $a_2$ ,  $\sigma_2$ , and  $n_2$  are those of the second group. Values higher than 2.5 are regarded as indicating a significant difference in the means.





7									
I.P.	Control	0	0	10	1.86	35.0	-1.86	-35.0	
Female, 52 yrs.	G	3.65	68.5	10	2.60	48.9	+1.05	+19.6	41
Morphine	GL	3.61	67.8	10	1.92	36.1	+1.69	+31.7	36
Wt., 53.2 kg									
Tot. protein									
6.63 gm/100 ml									
8									
I.P.	Control	0	0	7	1.95	35.5	-1.95	-35.5	
Female, 55 yrs.	G	3.81	69.8	10	4.05	73.6	-0.21	-3.8	77
Parkinsonism	GL	3.78	68.8	10	4.23	58.8	+0.55	+10.0	56
Wt., 55.0 kg	G	3.81	69.8	10	3.90	70.9	+0.06	-1.1	71
Tot. protein									
6.02 gm/100 ml									
9									
I.P.	Control	0	0	4	2.26	51.1	-2.26	-51.1	
Female, 55 yrs.	G	3.07	69.3	10	3.05	68.8	+0.02	+0.5	70
Mabuntion	GL	2.99	67.5	10	2.74	61.8	+0.25	+5.7	59
Wt., 41.3 kg	G	3.07	69.3	10	3.20	72.2	-0.13	+2.9	76
Tot. protein									
5.86 gm/100 ml									
10									
N.L.	Control	0	0	9	2.20	42.3	-2.20	-4.23	
Male, 38 yrs.	G	3.65	70.1	8	3.31	63.7	+0.34	+6.4	61
Mabuntion	GL	3.61	69.4	10	3.01	57.9	+0.60	+11.5	56
Wt., 52.0 kg									
Tot. protein									
1.11 gm/100 ml									

\* Nitrogen requirement for nitrogen equilibrium.

## DISCUSSION

The data from the first three balance experiments, like those from our previously reported studies, offer corroboration of the applicability of the method of Allison to human subjects with protein deficiency. The curves of nitrogen balance were rectilinear in the region of negative and low positive balance, as predicted by Allison. The K values for gluten were relatively constant, as were those for gluten plus lysine. On the other hand, the requirements of nitrogen for equilibrium were more variable, and, as in the previous studies, were dependent upon the amount of nitrogen excretion on a protein-free diet.

The findings in the remaining 7 experiments demonstrate that it is possible to obtain reasonably accurate K values for the Allison curves from only two points. The K values in 6 of these experiments were of the same order as those of the first three experiments. These results indicate that, at least for the comparison of one protein product with another in the same subject, it is unnecessary to go through the procedure of determining multiple points by a gradual increase in the nitrogen intake. Indeed, the determination of only two points may be even more reliable since, under the conditions of a relatively short experiment, the subjects can be kept on each regimen long enough for an accurate nitrogen balance assay. This scheme is essentially the same as that used by Mueller and Cox ('47), except that their experiments were carried out in normal subjects and that Allison's K values as such were not determined. Actually, one of the two points used for the curve need not be that for zero nitrogen intake; it could be that of any low nitrogen intake, provided the subjects were kept on that intake long enough for stabilization.

In each of the 10 balance experiments, the nitrogen balance index for gluten reinforced with lysine was appreciably higher than that for gluten alone. This result was of course to be expected, since numerous investigators have indicated that the limited nutritional value of wheat gluten can be enhanced by the addition of lysine. The earliest of these investigators

were Osborne and Mendel ('14). But they, as well as many more recent workers, based their conclusions on rat growth experiments, which are not necessarily applicable to man. Recently, Kuether and Myers ('48) have demonstrated the improvement in nitrogen balance in human subjects when cereal proteins were supplemented with lysine, as have Bricker, Mitchell and Kinsman ('45).

The average K value of 0.62 found in the present experiments is considerably higher than the 0.44 reported by Allison ('48) for a similar product fed to normal dogs. Allison in this communication does not report the value of the nitrogen balance index for gluten in protein-deficient dogs, but his data for other proteins indicate that the index is considerably increased when protein-depleted animals are utilized. Allison found that the K value rose from 0.44 to 0.82 in normal dogs when the gluten was reinforced with lysine; this elevation is considerably greater than that shown in the present experiments on human subjects with protein deficiency. The variance in these two sets of data may be due to species difference or possibly to an error in the K value for gluten. In both studies, at any rate, it has been demonstrated that the addition of lysine to gluten so increases the nutritive value of the product that it approaches or equals that of casein, which has been found by Allison to have a K value of around 0.80.

That the average nitrogen requirement for equilibrium using unfortified gluten was as low as 80 mg per kilogram per day was at first glance surprising. Such a value is equivalent to an intake of 35 gm of protein per day for a 70 kg person — a remarkably low requirement for what has been regarded as a relatively poor protein. However, it must be recognized that most, if not all, of our subjects were hospital patients with chronic protein deficiency. Such subjects have been shown to require a much smaller intake of nitrogen for nitrogen balance than normal individuals, unless the protein deficiency is associated with an acute "catabolic assault" such as trauma, infection, shock, or burn (Browne, Schenker and Stevenson, '44; Allison, '48; Hoffman et al., '48). The requirements for

nitrogen balance with wheat gluten feeding would undoubtedly have been much higher if the subjects had been normal and in good nutritional states. If the concentration of lysine in our wheat gluten is 1%,<sup>7</sup> and if the daily requirement of lysine in a 70 kg person is about 0.8 gm,<sup>8</sup> then the normal 70 kg subject would require about 80 gm of wheat gluten per day, or more than twice that required by our protein-deficient subjects. Such values are in keeping with the findings of Kuether and Myers ('48) and of Bricker, Mitchell and Kinsman ('45), the former utilizing whole wheat and the latter white flour.

The low requirements for nitrogen balance in our protein-deficient patients again illustrate the danger of judging the relative efficacy of any particular protein by comparing the amount required for balance in one group of subjects with that found for another protein by other investigators using a different type of subject. Nevertheless, the data imply that even a relatively poor protein is capable of producing appreciably positive nitrogen balances in severely depleted subjects, especially if properly fortified.

#### SUMMARY

In 10 hospital patients, most of whom had recognizable protein deficiency, a comparison was made of the nutritive value of wheat gluten alone and of the same product reinforced with 4% L-lysine.

The nitrogen balance index method, utilized by Allison in dogs, was found applicable to these human subjects. The nitrogen balance index, whether determined from the straight line curves drawn from multiple points or from only two points, tended to be constant for the particular protein preparation, whereas the nitrogen requirement for nitrogen equilibrium was more variable.

<sup>7</sup> Personal communication from Dr. Alfonse Walti, Interchemical Corporation, Biochemical Division, Union, New Jersey.

<sup>8</sup> The tentative requirement for lysine as determined by Dr. William C. Rose; personal communication.

The mean nitrogen balance index for gluten was 0.62. For gluten plus lysine, it was significantly higher, 0.76, approaching the value for casein. Thus lysine was shown to enhance the nutritive value of gluten for humans, as it had previously been shown to do for lower animals.

In patients with chronic protein deficiency even unreinforced gluten was found to be capable of producing positive nitrogen balances with moderate intakes.

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nitrogen balance with wheat gluten feeding would undoubtedly have been much higher if the subjects had been normal and in good nutritional states. If the concentration of lysine in our wheat gluten is 1%,<sup>7</sup> and if the daily requirement of lysine in a 70 kg person is about 0.8 gm,<sup>8</sup> then the normal 70 kg subject would require about 80 gm of wheat gluten per day, or more than twice that required by our protein-deficient subjects. Such values are in keeping with the findings of Kuether and Myers ('48) and of Bricker, Mitchell and Kinsman ('45), the former utilizing whole wheat and the latter white flour.

The low requirements for nitrogen balance in our protein-deficient patients again illustrate the danger of judging the relative efficacy of any particular protein by comparing the amount required for balance in one group of subjects with that found for another protein by other investigators using a different type of subject. Nevertheless, the data imply that even a relatively poor protein is capable of producing appreciably positive nitrogen balances in severely depleted subjects, especially if properly fortified.

#### SUMMARY

In 10 hospital patients, most of whom had recognizable protein deficiency, a comparison was made of the nutritive value of wheat gluten alone and of the same product reinforced with 4% L-lysine.

The nitrogen balance index method, utilized by Allison in dogs, was found applicable to these human subjects. The nitrogen balance index, whether determined from the straight line curves drawn from multiple points or from only two points, tended to be constant for the particular protein preparation, whereas the nitrogen requirement for nitrogen equilibrium was more variable.

<sup>7</sup> Personal communication from Dr. Alfonse Walti, Interchemical Corporation, Biochemical Division, Union, New Jersey.

<sup>8</sup> The tentative requirement for lysine as determined by Dr. William C. Rose; personal communication.

# INFLUENCE OF THYROID ON UTILIZATION OF VITAMIN A

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There are several reports in the literature indicating that large amounts of vitamin A have an antithyroid effect (Abelin et al., '26; Sadhu and Brody, '47; Schulze and Hundhausen, '39). The purpose of the present experiments was to test the converse hypothesis; namely, that thyroid influences the utilization of vitamin A. An experiment was set up concerned with liver storage of vitamin A in relation to thyroid activity in which it was assumed that the storage is an indirect measure of utilization; the lower the storage, the greater the depletion. If the thyroid hormone causes the destruction of vitamin A or increases the rate of its utilization, there should be decreasing vitamin A storage with increasing thyroid hormone activity.

Previously it has been indicated that adequate functioning of the thyroid gland is important for the conversion of carotene to vitamin A (Anderson and Soley, '38; Drill and Truant, '47; Escamilla, '42; Fasold and Heidemann, '33; von Euler and Klussman, '32; Johnson and Baumann, '47), although there has been some evidence to the contrary (Wiese et al., '47, '48).

Certain conclusions as to a relationship between vitamin A administration and thyroid activity seem fairly well established. These include: the delay in the metamorphosis of thyroxine-treated tadpoles to whom vitamin A is administered (Eufinger and Gottlieb, '33); the lessened elevation





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Certain conclusions as to a relationship between vitamin A administration and thyroid activity seem fairly well established. These include: the delay in the metamorphosis of thyroxine-treated tadpoles to whom vitamin A is administered (Eufinger and Gottlieb, '33); the lessened elevation

of the basal metabolic rate or of oxygen consumption in animals given vitamin A simultaneous with the administration of thyroxine or dessicated thyroid (Belasco and Murlin, '40; Chevallier and Baert, '34; Sadhu and Brody, '47); and the decrease in thyroid size in animals treated with the thyrotropic hormone (Fellinger and Hochstädt, '36), thyroxine or thiouracil (Sadhu and Brody, '47) after large doses of vitamin A.

However, the literature also contains many reports concerning a vitamin A-thyroid hormone relation which are conflicting. Reports disputing any interaction were made by Baumann and Moore ('39), who found no increase in the survival rate of animals given vitamin A and thyroxine simultaneously as compared with animals given thyroxine alone. Weslaw and Wroblewski ('39) reported obtaining the same results in counteracting thyroid effects by using sesame oil as the vehicle for the vitamin. Schneider and Widmann ('34) reported that depletion in glycogen stores in the liver brought about by hyperthyroidism as a result of the administration of thyrotropic hormone was not found if carotene or vitamin A was administered simultaneously, but Fasold and Peters ('33) and Steffen and Zois ('38) reported no such effect. Finally, Johnson and Baumann ('48) were unable to find an effect of thyroid on liver storage of vitamin A.

#### EXPERIMENTAL

Litters of Wistar strain albino rats raised in our laboratory on the stock diet of Bills et al. ('31), varying in age from 5 weeks to two months, were used. Each litter was split into three experimental groups: (1) control, (2) thyroidectomized, and (3) thyroxine-treated<sup>1</sup> animals. *Thyroidectomy was chosen as the means of achieving the hypothyroid state, since no question of a side reaction from a drug—e.g., one of the thiourea compounds—would be involved in the interpretation of results.* Four days prior to thyroidectomy or the ad-

<sup>1</sup> We wish to thank Dr. S. Sidney Newcomber of E. R. Squibb and Sons for his contribution of the crystalline thyroxine used in this experiment.

ministration of thyroxine, each animal, including controls, was given 1000 U.S.P. units of vitamin A in corn oil by stomach tube (Shay and Gruenstein, '46) and placed on the standard U.S.P. vitamin A-free diet. From this time on till the conclusion of the experiment neither vitamin A nor carotene was administered, because of the concept of increased absorption from the gastrointestinal tract in hyperthyroid states (Althausen and Wener, '37) and the necessity of the thyroid hormone for conversion of carotene to vitamin A (Anderson and Soley, '38; Drill and Truant, '47; Escamilla, '42; Fasold and Heidemann, '33). The thyroidectomized animals were also given 2.5 gm calcium chloride per 100 gm of the standard diet to avoid tetany due to the inadvertent extirpation of the parathyroid glands. Crystalline thyroxine was administered subcutaneously in a dose of 0.2 mg per 100 gm of body weight per day until termination of the experiment. The litters were sacrificed at the end of a 38- to 45-day period and the livers analyzed for vitamin A and carotene. The period chosen was based on preliminary experiments. It was long enough for change in thyroid activity to have effect but shorter than the time required for complete depletion of liver storage of A in any of the groups. The pituitaries of the thyroidectomized animals were examined histologically to ascertain whether thyroidectomy had been successful (Selye, '47). In all cases where histological evidence of thyroidectomy was absent, the animals were excluded from the series. Two animals out of a total of 12 were thus excluded.

Vitamin A determinations on the livers were done by the method of Sobel et al. ('48). The statistical analysis of the results was made utilizing Fisher's ('36) test of significance for small samples in which "P" represents the probability that the difference between the two means is due to chance. When P is 0.05 or less, the difference between the means may be considered statistically significant.

## DISCUSSION

From table 1 it can be seen that the mean vitamin A storage was highest in the thyroidectomized animals (43.4 ug/liver), intermediate in the thyroxine-treated animals (34.4 ug/liver) and lowest in the controls (22.8 ug/liver), while weight gain was highest in the controls (103 gm), intermediate in the thyroxine-treated animals (46 gm) and lowest in the thyroidectomized animals (36 gm); i.e. where the highest stores were obtained, the weight gain was least.

The differences in weight, comparing the control with the hyperthyroid group and the control with the hypothyroid group, were significant ( $P=0.0001$  in the former and  $P<0.001$  in the latter), but the comparison of the weight gain of the thyroidectomized with that of the thyroxine-treated animals was not significant ( $P=0.46$ ). However, the differences in vitamin A storage were all significant. ( $P$ , comparing controls with thyroxine-treated animals, was 0.03, for controls compared with thyroidectomized animals was  $<0.001$ , and for the thyroxine-treated compared with thyroidectomized animals was 0.05.)

On the basis of the results, one cannot attribute the vitamin A storage, to any great extent, to the direct action of the thyroid hormone. If this were to be the case, the thyroidectomized and the thyroxine-treated animals should show the extremes of storage and, as stated in the introduction, the hypothyroid animals should show the most and the hyperthyroid animals the least. However, a correlation can be seen in comparing storage to weight gain. The highest stores were found in the group showing the lowest weight gain, i.e. the thyroidectomized group, and the lowest stores in that group showing the greatest weight gain, i.e. the controls. It appears from these results that the presence or absence of thyroid influences storage to a large extent only to the degree that it affects growth. These results are in agreement with those of Johnson and Baumann ('48), although their experimental procedure differed in that the hypothyroid state was achieved by the administration of thiouracil and the hyper-

TABLE 1  
Vitamin A content of the livers of thyroxine-treated, thyroidectomized and normal rats<sup>1</sup>

GROUP	LITTER <sup>2</sup>	SEX	WEIGHT (GM)			VITAMIN A (μG/LIVER) <sup>3</sup>
			Initial	Final	Change	
THYROXINE	A	F	72	138	66	43.0
		M	71	128	57	38.0
	B	M	65	121	56	28.5
		F	39	62	23	40.5
	C	F	82	138	56	30.0
		M	81	124	43	31.0
	D	F	84	119	35	36.0
		F	80	132	52	28.0
Mean					46	34.4
THYROIDECTOMY	A	F	72	55	— 17	55.0
		F	60	140	80	41.0
		M	65	144	75	45.0
	B	M	69	84	15	47.5
		F	96	134	38	37.5
		M	99	150	51	59.5
	C	F	81	127	46	33.5
		F	78	111	33	32.5
	D	F	82	62	— 20	52.5
		M	81	136	55	29.5
Mean					36	43.4
CONTROL	A	M	58	203	145	25.0
		M	33	75	42	49.0
	B	F	60	199	139	18.0
		M	47	140	93	16.0
	C	F	86	170	84	21.0
		M	91	210	119	10.0
	D	F	77	184	107	24.0
		F	92	186	94	19.0
Mean					103	22.8
P <sup>4</sup>	Comparing thyroxine treated with thyroidectomized animals				0.46	0.05
P	Comparing controls with thyroidectomized animals				< 0.001	< 0.001
P	Comparing controls with thyroxine treated animals				0.0001	0.03

<sup>1</sup> Prior to thyroidectomy or administration of thyroxine, each animal, including controls, was given 1000 U.S.P. units of vitamin A in oil and subsequently kept on a vitamin A free diet.

<sup>2</sup> Each letter represents corresponding litter group.

<sup>3</sup> Carotene determinations were made on all livers, but the levels in all instances were negligible.

<sup>4</sup> P = probability that the difference between the two means is due to chance.

thyroid state by desiccated thyroid, and vitamin A was administered during the course of the experiment. On comparing weight gain and vitamin A storage in the thyroxine-treated and the thyroidectomized animals, the difference in storage is statistically significant (table 1) but the difference in weight is not. From this it appears that storage is affected in some way by the thyroid hormone; the greater the thyroid activity the lower the storage of vitamin A.

Even if one were to exclude these thyroidectomized animals which lost weight (table 1), the vitamin A stores of that group would still be greater (41.3 ug/liver) than those of the thyroxine-treated animals, although the weight gain (49 gm) would be about equal. While the difference in thyroid activity, comparing either of these two groups with the controls, might to some extent contribute to the differences in vitamin A stores, the growth effect is of so much greater influence that it completely masks any such secondary effect. Possibly, if an experiment were run using adult animals where growth effects would be minimum, this secondary effect would become more apparent. However, the secondary effect, as manifested in this experiment, is certainly not of the magnitude one would expect if the thyroid hormone influenced the destruction or utilization of vitamin A. Also, it cannot be definitely stated that this secondary effect is due to the direct action of the thyroid hormone on vitamin A, for it may be related merely to differences in metabolic activity.

#### SUMMARY

Vitamin A storage in the liver of rats on a vitamin A free diet was highest in the thyroidectomized animals, intermediate in the thyroxine-treated animals and lowest in the controls.

Growth of the control rats was most marked and probably accounts for the lower stores of vitamin A during a period when only endogenous vitamin A was available to the animals. Thyroid activity, in this instance, was indirect, in that growth was greatest in the euthyroid state.

Higher stores of vitamin A were found in thyroidectomized as compared with thyroxine-treated animals after a period of depletion, although there was no statistically significant difference in the weight changes. However, this cannot be necessarily ascribed to a direct thyroid-vitamin A relationship.

#### ACKNOWLEDGMENT

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# VITAMIN STUDIES IN MIDDLE-AGED AND OLD INDIVIDUALS

## III. THIAMINE AND PYRUVIC ACID BLOOD CONCENTRATIONS <sup>1</sup>

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### THREE FIGURES

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### INTRODUCTION

The data presented in this publication were collected with the purpose of establishing the fasting thiamine and pyruvic acid blood concentrations of individuals of various ages. The study comprised 220 individuals (117 men and 103 women) between the ages of 40 and 102 years. For comparison, 29 younger individuals (13 men and 16 women) were included in the investigation. The majority of the older subjects were inmates or patients in the St. Louis City Infirmary and City Infirmary Hospital, whereas the greater part of the younger subjects were private volunteers. The study was undertaken during the months from December, 1947, to May, 1948. During this period the diet offered the patients in the above-mentioned institutions was calculated to contain an average of 2,150 cal., 265 gm of carbohydrate, and 1.9 mg of thiamine daily, a vitamin content which is considered adequate with regard to the caloric intake (Cowgill, '34). The caloric value and thiamine content of the diets of the younger subjects were not evaluated. None of the individuals studied had re-

<sup>1</sup>Funds and materials provided by Hoffmann-La Roche, Inc.

ceived vitamin preparations prior to the blood analysis. Individuals with febrile diseases or carcinoma, and patients suffering from diabetes mellitus, diabetes insipidus, liver cirrhosis, nephritis, pernicious anemia, leukosis and organic gastric and intestinal lesions were not included in the study.

#### METHODS

The thiamine determinations were performed on whole blood by the method of Friedemann and Kmieciak ('43) using a sensitive fluorometer.<sup>2</sup> The blood was collected in the morning from the fasting and resting individuals, who had received no quinine, salicylates or other fluorescing preparations during the 24 hours preceding the thiamine determination. In the first 80 analyses duplicate blood samples were carried through the whole procedure; two aliquots of each eluate were oxidized and the thiochrome determined fluorometrically, thus giving a total of 4 readings for each blood sample. The average difference between the mean values of the two samples from the same blood was 0.25  $\mu\text{g}\%$ , or 7.6%, and between the two aliquots of the same eluate 0.23  $\mu\text{g}\%$ , or 7.0 %. After the reliability of the technique had thus been established, the subsequent analyses were performed on single blood samples with duplicate fluorometric readings on the eluate. Thiamine added to the blood was recovered quantitatively, or nearly quantitatively (92 to 103%).

#### *Determination of blood pyruvic acid*

For pyruvic acid blood determinations the procedure of Fabricsius-Hansen ('48) was used, which is based on the methods of Lu ('39), Bueding and Wortis ('40), and Carlström, Myrbäck, Holmin and Larsson ('39). This procedure, which has previously been tested in the laboratory of one of the present authors, has proved its reliability and permits quantitative recovery of pyruvic acid added to the blood. The

<sup>2</sup> The fluorometer was constructed by the Photovolt Corporation, New York City, and was specially adapted in our laboratory for thiochrome estimations.

method is rather laborious, as usually 24 to 28 individual extraction procedures are required in each analysis for isolation of the pyruvic acid hydrazone, but several samples may conveniently be carried through at the same time.

A few modifications of the technique were found advantageous under our present working conditions. Thus, separatory funnels instead of ordinary tubes were used for separation of the layers and ether was substituted for ethyl acetate. Also a Coleman spectrophotometer was employed for the final color estimation in place of the step photometer used by Fabricius-Hansen. For a discussion of the method readers are referred to the original publication. The average difference found between duplicate analyses in the present series of determinations was 0.06 mg% or 5%.

#### EXPERIMENTAL

##### *Fasting blood thiamine concentration*

In figure 1 the fasting thiamine blood values are plotted graphically in relation to the age of the individuals. Table

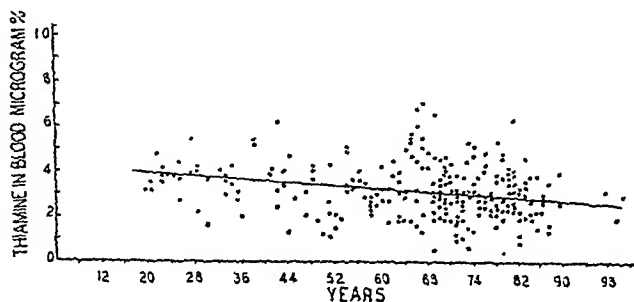


Figure 1

1 contains the summarized values for the age groups 16-39, 40-59, 60-69, 70-79, and 80-102 years, and the values for men and women separately. It will be seen from the data that the values show great variation among individuals and that there appears to be a slight tendency to a lowering of the blood values with advancing years. This is suggested by a

TABLE 1  
*Fasting thiamine and pyruvic acid in blood*

AGE GROUP	NO.	THIAMINE IN BLOOD		S.D. <sup>1</sup>	OBSERVATIONS BELOW 2 $\mu$ g %		NO.	PYRUVIC ACID IN BLOOD		S.D. <sup>1</sup>
		Mean	Range		no.	%		Mean	Range	
		$\mu$ g %	$\mu$ g %			%		mg %	mg %	
16-39	Men	3.8	2.2-5.3	0.7	0	0	13	1.2	0.9-1.6	0.3
	Women	3.8	2.0-5.5	1.0	0	0	15	1.1	0.3-1.9	0.5
	Total	3.8	2.0-5.5	0.9	0	0	28	1.1	0.3-1.9	0.4
40-59	Men	3.4	1.4-6.3	1.3	1	5	17	1.1	0.4-1.7	0.4
	Women	3.1	1.2-5.1	1.1	4	17	24	0.9	0.2-1.8	0.4
	Total	3.3	1.2-6.3	1.2	5	12	41	1.0	0.2-1.8	0.4
60-69	Men	3.6	0.7-7.2	1.5	3	10	18	1.2	0.5-2.8	0.3
	Women	4.0	1.9-7.0	1.5	1	5	17	0.9	0.5-1.5	0.3
	Total	3.7	0.7-7.2	1.5	4	8	35	1.0	0.5-2.8	0.3
70-79	Men	3.3	0.8-7.2	1.2	5	12	25	1.1	0.8-2.1	0.3
	Women	3.1	1.2-5.3	1.0	2	7	29	1.0	0.3-1.7	0.4
	Total	3.2	0.8-7.2	1.1	7	10	54	1.1	0.3-2.1	0.3
Above 80	Men	3.0	0.6-4.8	0.9	4	14	12	1.1	0.5-1.7	0.3
	Women	3.2	1.0-6.5	1.2	3	10	29	1.2	0.4-1.9	0.3
	Total	3.1	0.6-6.5	1.1	7	12	41	1.1	0.4-1.9	0.3

<sup>1</sup> Standard deviation of the mean.

moderate slope of the line representing the mean thiamine values in the different age groups in figure 1, and by the fact that 8 to 12% of the individuals above 40 years of age showed blood values below 2.0  $\mu\text{g}\%$ , whereas values below this level were not encountered in the younger subjects. That the tendency to a reduction of the thiamine blood value with age is only slight is exemplified by a calculation of the coefficient of correlation between age and the thiamine blood concentration, which was found to be  $-0.20$ . The slight reduction in

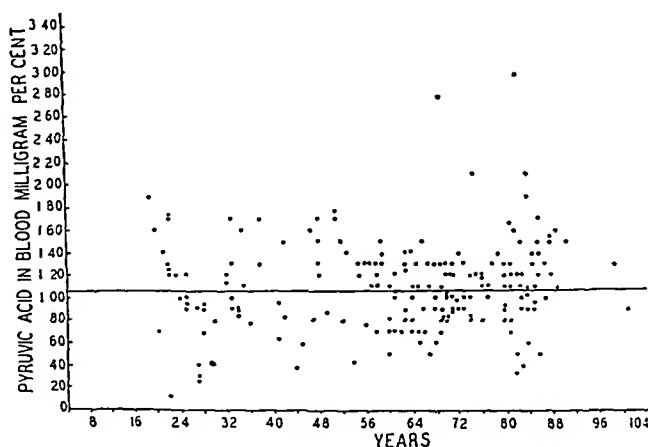


Figure 2

blood thiamine concentration may be due, in part at least, to the greater frequency of anemia with age, the red blood cells containing a higher thiamine value than the plasma. The mean thiamine blood value for the 249 subjects examined was 3.4  $\mu\text{g}\%$ . No difference was observed between the thiamine values in men and women.

#### *Fasting blood pyruvic acid concentration*

In figure 2 the fasting pyruvic acid blood values for 199 individuals are plotted graphically in relation to the age of the subjects. The values show a great variation among individuals but fail to reveal any effect of age or sex on the

pyruvic acid blood concentration. The mean pyruvic acid value found was 1.1 mg%.

*Correlation between fasting thiamine and fasting pyruvic acid blood values*

The correlation between the fasting thiamine and fasting pyruvic acid blood values was considered to be of special interest in view of observations in the literature on the occurrence of elevated blood pyruvic acid concentrations in instances of thiamine deficiency. In figure 3 the corresponding

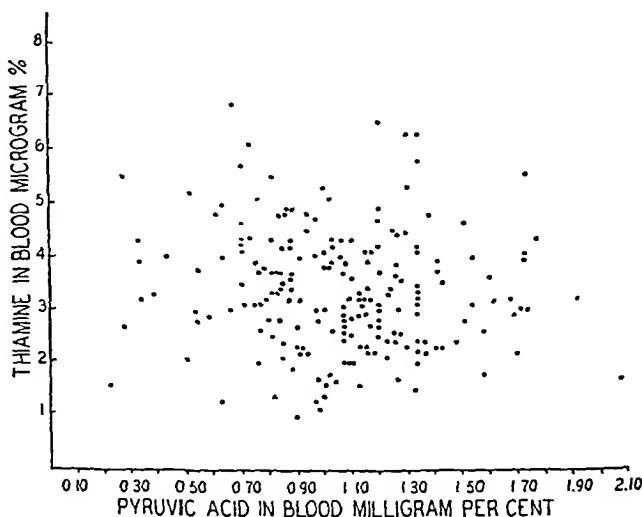


Figure 3

values have been plotted graphically. As will be seen, no definite correlation can be observed, a fact which is also revealed by a calculation of the coefficient of correlation between the thiamine and pyruvic acid values, which was found to be  $-0.14$ .

DISCUSSION

The thiamine blood values reported in this paper are somewhat lower than the values observed by Friedemann and Kmieciak ('43) in examination of 36 adults, using the same analytical

procedure (mean value in this investigation,  $3.4 \mu\text{g}\%$ ; in Friedemann and Kmiecik's study,  $5.7 \mu\text{g}\%$ ), but agree quite well with the few analyses reported by Foltz, Barborka and Ivy ('44), who likewise employed the thiochrome method. As a general rule investigators using a microbiological technique have found higher values for vitamin  $B_1$  in blood than have been observed in studies using the chemical determination, a fact which has been discussed by Hennessy ('47). Oldham, Davis and Roberts ('46), using a microfermentation procedure in an investigation of 11 young women, observed thiamine concentrations of between  $4.0$  and  $6.7 \mu\text{g}\%$ , with an average value of  $5.2$ . The most extensive studies on this subject have been reported by Bang ('44), who examined 38 normal, non-institutionalized individuals below the age of 70, using the phycomyces growth method. The mean thiamine value observed was  $7.9 \mu\text{g}\%$  ( $5.0$  to  $12.4 \mu\text{g}\%$ , s.d.  $2.0$ ); no certain correlation was found between the age and the blood thiamine concentration. In 13 institutionalized individuals above 70 years of age slightly lower thiamine values were encountered. These findings, apart from the higher absolute values, are in good agreement with the observations reported in the present paper.

#### SUMMARY

An investigation was made of the concentrations of blood thiamine and pyruvic acid in 220 middle-aged and old individuals offered a diet adequate in vitamin  $B_1$ , and in 29 younger subjects. The average concentration of thiamine was  $3.4 \mu\text{g}\%$  (s.d.  $1.1 \mu\text{g}\%$ ) and of pyruvic acid  $1.1 \text{mg}\%$  (s.d.  $0.3 \text{mg}\%$ ). A slight tendency was noted for the thiamine value of blood to decrease with age (coefficient of correlation age/thiamine,  $-0.20$ ), whereas the pyruvic acid level showed no change with advancing years. No significant correlation was observed between the thiamine and pyruvic acid values in the same individuals (coefficient of correlation thiamine/pyruvic acid,  $-0.14$ ).

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# STUDIES ON THE COMPARATIVE NUTRITIVE VALUE OF FATS

## XI. ON THE POSSIBLE GROWTH-PROMOTING ACTIVITY OF $\Delta^{12}$ -OCTADECENOIC ACID<sup>1</sup>

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A number of recent investigations have failed to confirm the reputed growth-promoting action of vaccenic acid which was postulated by Boer, Jansen and Kentie ('47). These workers presented data indicating that "summer butter" contains a substance not found in "winter butter" or vegetable oils, and which has specific growth-promoting properties when tested on rats. The compound responsible was tentatively identified as vaccenic acid ( $\Delta^{11}$ -octadecenoic acid), since it was believed to be identified with the  $\Delta^{11}$ -elaidic acid previously described by Bertram ('28). It was shown by Boer, Jansen, Kentie and Knol ('47) that a similar stimulation in growth could be produced when the diet was supplemented with vaccenic acid prepared from hydrogenated China-wood oil. The fatty acid was admittedly not a pure fatty acid, as indicated by lower than theoretical iodine number and melting point.

<sup>1</sup>This work was carried out under a research grant from The Best Foods, Inc. The authors wish to acknowledge the helpful advice of Professor Anton J. Carlson of the University of Chicago, Professor Arthur W. Thomas of Columbia University, and Dr. H. W. Vahlteich of The Best Foods, Inc., during the course of the experiments. Contribution number 210 from the Department of Biochemistry and Nutrition, University of Southern California.

Opposite results from those of Boer, Jansen and Kentie ('47) were obtained by Deuel et al. ('48b) when supplements of a highly purified vaccenic acid prepared by the method of Boeseken et al. ('30) were fed to rats. A specimen of hydrogenated China-wood oil also produced no stimulatory effect. It was suggested that the low growth response obtained with rapeseed oil as compared with butterfat might be partially attributable to the poor digestibility of the latter fat in rats (Deuel, Cheng and Morehouse, '48a). Euler, Euler and Linderman ('48) also reported that vaccenic acid produces no augmentation in growth of rats on a rapeseed oil or margarine fat diet. The latter workers found that growth on rapeseed oil was markedly lower than on margarine fat, just as Deuel et al. ('48b) found to be the case when the growth on a rapeseed oil diet was compared with that on cottonseed oil or butter diets. Recently, Nath et al. ('48) have further confirmed these findings by demonstrating that vaccenic acid isolated from natural sources or prepared synthetically as the *cis*- or *trans*- form causes no growth-stimulating effect on corn oil diets.

Although in this laboratory we have consistently been unable to demonstrate any superior growth-promoting activity of summer butter over that of vegetable fats (Deuel et al., '44), the possibility still exists that the results of Boer, Jansen and Kentie ('47) may have been obtained with an unsaturated fatty acid other than vaccenic acid. The latter acid might readily have been confused with  $\Delta^{12}$ -octadecenoic acid, which has a melting point almost identical with that of vaccenic acid (39.5°C., Richter, '42, as contrasted with 39.7-40.1°C.) and the same iodine number. Rao and Daubert ('48) have, however, obtained a value of 42.5°C. for the melting point of vaccenic acid separated from beef tallow. The importance of  $\Delta^{12}$ -octadecenoic acid from a nutritional standpoint is further emphasized by the fact that its presence has been reported in hydrogenated fats (Markley, '47; Ralston, '48).

The present experiments were undertaken to determine whether  $\Delta^{12}$ -octadecenoic acid possesses any growth-stimulating activity. For this purpose, a sample of *trans*- $\Delta^{12}$ -octadecenoic acid was available; tests were also carried out with a *cis-trans*-mixture of  $\Delta^{11}$ - and  $\Delta^{12}$ -octadecenoic acids, as well as a 50-50 mixture of *trans*- $\Delta^{11}$  and  $\Delta^{12}$ -octadecenoic acids. Information concerning the preparation and proof of structure of these acids is included in the appendix.

#### EXPERIMENTAL

The tests were carried out with weanling rats from our stock colony which were 28 days old. Littermates were evenly distributed among the 5 experimental groups. Experiments were carried out with both male and female rats. Sixteen rats (8 male, 8 female) were used in each group. The animals were kept in individual cages with raised screen bottoms. Food and water were given ad libitum but the amount of food consumed was measured. Diets were prepared weekly and were fed on alternate days. The reserve food was kept in the refrigerator between feedings.

The diets were similar to those used earlier in the vaccenic acid tests (Deuel et al., '48b). The basal diet consisted of ground whole wheat, 72 parts; commercial casein, 5 parts; yeast,<sup>2</sup> 10 parts; salt mixture (Osborne-Mendel, '17), three parts; and fat,<sup>3</sup> 10 parts. The fats used, and the supplements for the various experimental groups, were as follows: group I, butterfat<sup>4</sup> and no supplement; groups II to V, cottonseed oil<sup>5</sup> with no supplement (II), with 20 mg of  $\Delta^{12}$ -*trans*-octadecenoic acid (III), with 40 mg of a mixture of  $\Delta^{11}$ - and  $\Delta^{12}$ -*trans*-octadecenoic acids (IV), and with 40 mg of a mixture of *cis*- and *trans*- $\Delta^{11}$  and  $\Delta^{12}$ -octadecenoic acids (V). The

<sup>2</sup> Anheuser-Busch, strain G.

<sup>3</sup> The following supplements were added to all oils per 100 gm oil: carotene (General Biochemicals, Inc.) 2.4 mg; fish liver oil containing 350,000 I.U. of vitamin A per gram, 19.2 mg; crystalline vitamin D<sub>2</sub>, 0.05 mg;  $\alpha$ -tocopherol, 36.0 mg; commercial butter flavor, 1.2 mg.

<sup>4</sup> Knudsen brand, sweet cream butter, obtained locally.

<sup>5</sup> Wesson oil.

TABLE 1

Summary table of mean starting weights, weight gains, and food consumption of rats receiving a diet containing butterfat (I) cottonseed oil (II), cottonseed oil plus trans- $\Delta^{12}$ -octadecenoic acid (III), cottonseed oil plus  $\Delta^{11}$ - and  $\Delta^{12}$ -trans-octadecenoic acids (IV), or cottonseed oil plus trans- and cis-mixture  $\Delta^{11}$ - and  $\Delta^{12}$ -octadecenoic acids (V). Each group contained 16 rats (8 males, 8 females).

DIET NUMBER	STARTING WEIGHT AT 28 DAYS OF AGE			GAIN DURING 7 WEEKS			FOOD INTAKE IN 6 WEEKS		
	Male <sup>1</sup>	Female <sup>1</sup>	Average	Male <sup>1</sup>	Female <sup>1</sup>	Average	Male	Female	Average
	gm	gm	gm	gm	gm	gm	gm	gm	gm
I	47.9 $\pm$ 2.1	50.3 $\pm$ 2.3	49.1	175.3 $\pm$ 8.3	124.3 $\pm$ 5.5	149.8	581.9	553.3	567.6
II	49.0 $\pm$ 2.6	49.9 $\pm$ 2.6	49.5	181.9 $\pm$ 9.6	119.1 $\pm$ 3.3	150.5	569.2	515.1	542.2
III	50.9 $\pm$ 2.6	48.3 $\pm$ 2.4	49.6	169.5 $\pm$ 12.0	119.8 $\pm$ 4.1	145.7	565.9	525.3	546.6
IV	49.6 $\pm$ 2.0	47.3 $\pm$ 2.2	48.5	182.5 $\pm$ 16.5	123.0 $\pm$ 4.6	152.8	586.7	499.9	543.8
V	49.1 $\pm$ 2.5	51.8 $\pm$ 2.9	50.5	181.6 $\pm$ 8.7	110.1 $\pm$ 3.9	145.9	611.1	527.6	569.4

<sup>1</sup>Including the standard error of the mean calculated by the formula  $\sqrt{\sum d^2/n - 1}/\sqrt{n}$ , where "d" is the deviation from the mean and "n" is the number of observations.

supplements were dissolved in cottonseed oil so that the desired amount was present in 0.1 ml, and were administered to the rats daily.

The results for the 5 groups of rats are summarized in table 1.

#### DISCUSSION

No stimulating effect was noted on the growth of rats when  $\Delta^{12}$ -octadecenoic acid or a mixture of  $\Delta^{11}$ - and  $\Delta^{12}$ -octadecenoic acids were administered to animals receiving a cottonseed oil diet. This would indicate that no special growth-promoting action can be ascribed to the unsaturated  $\Delta^{12}$ -octadecenoic acid. The result of Boer, Jansen and Kentie ('47) apparently cannot be due to the presence of the  $\Delta^{12}$ -octadecenoic acid in butterfat, which might readily have been an unsaturated acid mistaken for vaccenic acid.

Turpeinen ('38) also carried out tests with the ethyl ester of  $\Delta^{12}$ -octadecenoic acid prepared from 12-hydroxystearic acid. When 100 mg of a 1:2 mixture of the *cis*- and *trans*-isomers were fed daily to rats on a fat-free diet, no cure for the "fat deficiency" syndrome could be noted;  $\Delta^{12}$ -*cis*-octadecenoic acid was also found to be ineffective in curing this deficiency when fed in 100 mg doses.

The present experiments also confirm the earlier ones of Deuel et al. ('44, '48a, '48b) in showing that an identical growth obtains when the dietary fat is butterfat or cottonseed oil. Thus the average gain in weight over the 7-week period was 149.8 gm with the butterfat group and 150.5 gm with the cottonseed oil group. These experiments would seem to answer in the negative the question as to whether  $\Delta^{12}$ -octadecenoic acid possesses any specific nutritive properties. They further confirm this negative answer by demonstrating identical growth on butterfat and cottonseed oil diets.

#### SUMMARY

1. No increase in growth over that obtained with the unsupplemented diets could be noted in rats fed diets containing cottonseed oil and supplemented with  $\Delta^{12}$ -*trans*-octa-

decenoic acid, a 1:1 mixture of  $\Delta^{11}$ - and  $\Delta^{12}$ -*trans*-octadecenoic acids, or a mixture of *cis*- and *trans*- $\Delta^{11}$ - and  $\Delta^{12}$ -octadecenoic acids.

2. Identical growth was obtained whether the diets contained summer butterfat or cottonseed oil.

3. The tests further confirm the inactivity of vaccenic acid ( $\Delta^{11}$ -octadecenoic acid) as a dietary supplement.

#### APPENDIX

##### *Preparation of acids (Gooding and Brown)*

The acids to be described were prepared with the intention of comparing them with the solid unsaturated acids of butterfat. It was also our intention to identify more positively the acid of butter, since it appeared that vaccenic acid might have been confused with the closely similar *trans*-12-octadecenoic acid in view of the poor degree of purity reported for the naturally-occurring acid. This latter objective has not been achieved, since we have been unable to resolve contradictory results of oxidation and ultimate analyses. However, we may note that the acids from butter are of the *trans* configuration, since their infrared absorption spectra are very similar to, if not identical with, that of vaccenic acid isolated from beef tallow and reported by Rao and Daubert ('48), and also that of synthetic vaccenic acid reported by Ahmad, Bumpus and Strong ('48). The absorption curve of the butter acid is also closely similar to that of our  $\Delta^{12}$ -octadecenoic acid described below.<sup>a</sup>

From Texas farm butter there was obtained by means of lead salt and mercury salt methods, combined with distillation and crystallization from MeOH, a material of m.p. 35–35.5°C., I.V. 85.0 and eq. wt. 287.5, and from New York dairy butter a material of m.p. 34.7–36.3°C. and I.V. 84.0. Repeated crystallization of the latter product (10 gm) from acetone gave 1.7 gm of acid of m.p. 41–41.5°C. and I.V. 86.2. Ahmad et al. ('48) have reported m.p. 43–44°C. and I.V. 87.3 for synthetic vaccenic acid.

One thousand grams of hydrogenated castor oil (I.V. 1.7) was dehydrated (Fokin, '12) under reduced pressure by heating for three hours at 260°C. with 1% naphthalene sulfonic acid. The resulting product was converted to methyl esters by methanolysis, water-washed, dried and distilled. The ester mixture of I.V. 69.0 was

<sup>a</sup> We are indebted to Dr. H. T. Beans, Professor Emeritus of Columbia University, for the determination of the infrared absorption spectra on these compounds.

twice partially solidified by chilling to decrease the stearate and OH-stearate content, by which means the I.V. of the final filtrate was increased to 75.1.

The liquid esters were saponified with KOH, neutralized with glacial acetic acid, and the lead salts insoluble in SD 30 alcohol were filtered by suction using a rubber dam. The lead soaps were washed with cold water before being converted by boiling with 1:1 HCl to acids of 70.4 I.V. The Bertram mercury salt method was used to separate the solid unsaturated acids from the saturated acids. The acids recovered from the filtrate, designated as *trans*-12-octadecenoic (I.V. 85.0, m.p. 33.5–37.5°C., S.V. 199.0, A.V. 172), were oxidized by  $\text{KMnO}_4$  in acetone. The dicarboxylic acid fraction had an eq. wt. of 116 [theoretical for  $(\text{CH}_2)_{10}(\text{COOH})_2$ , 115] and a m.p. of 94–101°C. A later purification by crystallization from diluted acetone increased the I.V. to 87.3°C. and the m.p. to 40–41°C.

A mixture of *trans*-11 and *trans*-12  $\text{C}_{18}$  acids was prepared by isomerization of the liquid unsaturated acids recovered from the filtrate from the first lead soap separation following the dehydration described above, by heating with 0.5% Se powder for 5 hours at 210°C. Solid unsaturated acids were separated from liquid unsaturated acids by the lead soap method. The distilled, bleached, and steam-deodorized solid unsaturated acids had the following values: I.V. 86.7, m.p. 34.5–36.0°C., A.V. 198.7, and S.V. 198.8. A portion was oxidized to yield a dicarboxy acid of m.p. 96–103°C. and eq. wt. 110.5, corresponding to that which result from the oxidation of a mixture of approximately 65% of  $\Delta^{11}$  and 35% of  $\Delta^{12}$  acids.

The dehydration of hydrogenated castor oil was repeated and the methyl esters distilled and saponified to obtain a mixture of acids of I.V. 75.0, A.V. 189, S.V. 198.7. This product was assumed to be a mixture of *cis*- and *trans*-  $\Delta^{11}$  and  $\Delta^{12}$  acids, with a small proportion of stearic acid and corresponding methyl esters.

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# STUDIES ON THE COMPARATIVE NUTRITIVE VALUE OF FATS

## XII. THE DIGESTIBILITY OF RAPESEED AND COTTONSEED OILS IN HUMAN SUBJECTS<sup>1</sup>

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Variations in the digestibility of several fats in rats as compared with man have recently been reported. Thus, the coefficient of digestibility of hydrogenated peanut or corn oils melting at 50°C. was found to be 92.0 and 88.5, respectively, in man, although a higher melting hydrogenated peanut oil (m.p., 52.4°C.) had a digestibility coefficient of 79 (Holmes and Deuel, '21; Deuel and Holmes, '22). Blended hydrogenated cottonseed oil melting at 50°C. was digested to the extent of 87%, while blended hydrogenated peanut and corn

<sup>1</sup> This work was done under a contract between the University of Southern California and the Research and Development Branch, Military Planning Division of the Office of the Quartermaster General, U. S. Army.

This paper reports research undertaken in cooperation with the Quartermaster Food and Container Institute for the Armed Forces, and has been assigned number 247 in the series of papers approved for publication. The views or conclusions contained in this report are those of the authors. They are not to be construed as necessarily reflecting the views or indorsement of the Department of the Army.

Contribution no. 209 from the Department of Biochemistry and Nutrition, University of Southern California.

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<sup>3</sup> We wish to thank Mr. W. R. Harriman, Director, and Dr. Douglas W. Ritchie, Medical Director, Rancho Los Amigos, for making arrangements for this study.

oils melting at 51.1 and 54°C. were found to be digested to the extent of 92.8 and 91.5%, respectively, in tests on human subjects (Deuel and Holmes, '22). In distinction to these results on man, hydrogenated cottonseed oil melting at 46°, 54°, and 65°C. was found to be digested in the rat to the extent of only 83.8, 68.7, and 24.0%, respectively (Augur et al., '47). Hydrogenated lards melting at 55° and 61°C. had digestibility coefficients of 63.2 and 21.0, respectively (Crockett and Deuel, '47).

Another example of divergent results between man and rats has been noted with rapeseed oil. Although Holmes ('18) reported a digestibility coefficient of 98.8 for 4 male subjects who consumed an average of 82 gm of rapeseed oil daily, Deuel et al. ('48) found that crude rapeseed oil was digested only to the extent of 77% and that the refined oil was 82% utilized by rats.

There are several possible explanations for these apparent discrepancies between fat digestibility in man and rats. The first suggestion is that a species difference actually exists. An example of species variability which is well known is in the response to castor oil. In distinction to the cathartic action produced by castor oil in man, the rabbit, the guinea pig, and sheep are able to utilize this fat to a high degree and no intestinal upset results (McCay and Paul, '38; Paul and McCay, '42).

A second possibility in explaining the variable results is that different procedures were employed in the tests. In the experiments on man, account was taken only of the ether-soluble fraction of the feces, which would include the neutral fatty, fatty acid, and nonsaponifiable components. In the tests on rats, the fat excreted as soaps was also considered. The inclusion of the soap fraction does not change the calculated digestibility of such low-melting fats as margarine fat, commercial hydrogenated cottonseed oil,<sup>4</sup> prime steam lard, and bland lard since the soap content is low and the correction for metabolic soap compensates for this factor.

<sup>4</sup> Crisco.

However, this is not the case with the higher melting fats. Without considering the soap fraction, a hydrogenated lard sample melting at 55°C. was calculated to be digested to the extent of 97.6%; actually, when the soap figure was included, the fat was found to be utilized only 63.2% (Crockett and Deuel, '47). In another hydrogenated lard sample melting at 61°C., these same authors report a coefficient of digestibility of only 21; had the soap fraction not been considered, the calculated value would have been 88.7.

The present experiments were undertaken to determine whether rapeseed oil actually is as completely digested in man as the earlier tests indicate or whether the inclusion of the soap fraction of the feces in the computation would alter the conclusions. Cottonseed oil was included for comparison as it is an oil generally considered to be highly digestible in man and one which has been reported to be highly utilized by rats.

#### EXPERIMENTAL

The experiments were carried out on 8 volunteer subjects who were patients at the Rancho Los Amigos, which is one of the units for the chronically ill of the Los Angeles County Hospital System. Some of the subjects were the same individuals who had served in earlier studies where a comparison of the nutritive value of fresh and dehydrated foods was made (Deuel et al., '47). They were not suffering from any ailments which would be expected to alter their digestive processes or their nitrogen metabolism. The subjects were confined to a single ward which was used exclusively for the test. The other conditions of the test are similar to those reported earlier (Deuel et al., '47).

Three different menus were employed, which were repeated in rotation over the experimental periods. They were identical in the rapeseed and cottonseed oil tests except that the oil used in the food preparation varied with the oil under consideration. The composition of the menus and the amount of each food served are included in table 1.

About 88% of the fat consisted of the test fat, and the balance was made up of the lipid components of the low-fat diet. The average daily protein intake was calculated at 66.0 gm and of fat at 58.4 gm, of which 51.6 gm was the dietary fat under investigation. The average caloric value of the diets

TABLE 1

*The composition of the different menus employed in the fat digestibility tests*

BREAKFAST		LUNCH		DINNER	
Food	Amt.	Food	Amt.	Food	Amt.
	gm		gm		gm
Menu I					
Strained orange juice	183	Mashed potatoes	166	Vegetable soup	157
Cornflakes	16	Gravy	65	Cottage cheese	100
Graham date gems	89	String beans	150	Peach (half)	100
Sugar	22	Hot rolls	58	Lettuce	20
		Lettuce with	50	Mayonnaise	15
		French dressing	10	Bread	30
		Apricot tapioca	161	Prune whip	112
Menu II					
Strained orange juice	183	Spanish rice	176	Cottage cheese	100
Rice Krispies	30	Glazed carrots	113	Apricots	40
Cinnamon rolls	65	Cornbread	65	Orange	60
Sugar	22	Lemon sponge		Pear in syrup	60
		pudding	116	Lettuce	20
				Mayonnaise	10
				Muffins	53
				Brown sugar tapioca	112
Menu III					
Prunes	100	Chili and beans	207	Creamed peas on	164
Cornflakes	16	Raw chopped onions	20	Steamed potatoes	150
Bread	48	Bread	48	Sliced tomato	100
Sugar	18	Lettuce	50	Cornbread	90
		Beets	30	Pear in Jello	152
		Celery	25		
		Green pepper	8		
		French dressing	10		
		Shortcake	70		
		Cherries	50		

Additional food served: all meals — skimmed milk, 200 gm; breakfast — black coffee, 180 gm.

was 2,028 cal. and the daily food average 2,141 gm. One subject (Brown) received one-fourth additional portions in both tests, while the remaining 7 subjects received the quantities of foods listed in table 1.

The diets employed were extremely appetizing and were eaten with considerable relish. It was believed that such a dietary regime is more natural and avoids the considerable monotony that was the case with the blanc mange diets employed in the extensive series of tests at the U. S. Department of Agriculture (Langworthy, '23). Excellent subject cooperation can be obtained over long periods of time by the use of such a varied diet schedule. It does have the objection that small amounts of other fats are included in the basic diet. However, since these are apparently almost completely digested, they are not believed to influence the behavior of the predominant fat. The rolls, muffins, cornbread, shortcake, and gems had a considerable amount of the experimental fat incorporated in them, as did the French and mayonnaise dressings. These foods were as acceptable irrespective of whether rapeseed or cottonseed oil was used in their preparation.

The rapeseed oil was a commercial sample<sup>5</sup> which was refined by bleaching and deodorizing.<sup>6</sup> The refined oil had a light yellow color, a satisfactory odor, and a free fatty acid content of only 0.01%. The following constants were also found: saponification number, 167.7; iodine number, 96.3. It was the same product employed in the earlier tests on rats (Deuel et al., '48). The cottonseed oil used was a commercial oil.<sup>7</sup>

The methods of urine analysis were similar to those employed earlier (Deuel et al., '47), while the estimation of the fecal fat (neutral fat, fatty acid and soap fractions) was similar to that employed by Augur et al. ('47). No markers were used in the separation of feces. Since the subjects

<sup>5</sup> Obtained from the Pacific Vegetable Oil Company through the courtesy of Mr. M. G. Barradas of The Best Foods, Inc., San Francisco, California.

<sup>6</sup> We wish to thank Mr. R. H. Neal of the Bayonne Laboratories of The Best Foods, Inc., for refining the rapeseed oil.

<sup>7</sup> Wesson.

were accustomed to having a bowel movement each morning, the stool sample on the day of the test was rejected but the remaining stool samples during the experiments, including that excreted on the morning following the termination of the diet, were used for analysis. Because of the relatively long period of the test on each fat (9 days), the inaccuracy due to separation is considered to be minimum. The amount of fat consumed was estimated on the basis of the amount used in the preparation of the food.

The calculation of the digestibility coefficient was made by the usual procedure, making allowance for metabolic fat. Instead of using the correction factor of 9.89% of the weight of the dried stool (Langworthy and Holmes, '15), which is based only on the metabolic neutral fat, fatty acid, and non-saponifiable components excreted, a new figure of 19.8% was employed for total metabolic lipids. This was calculated on the assumption that the neutral fat, fatty acid:soap ratio was the same as the ratio between these substances in the current tests. The soap makes up about 50% of the total fecal fat in the 16 tests reported here.

## RESULTS

The results for the rapeseed oil tests are included in table 2, while those for the cottonseed oil experiments are given in table 3.

The average digestibility of rapeseed oil was 99.0%, while the figure for the cottonseed oil was 96.5%. Seven of the 8 subjects consumed 525.6 gm of fat over the 9-day period, of which 464.8 gm consisted of the fat under investigation. The 8th subject, who consumed 25% more of the diet, ate 657 gm of fat, of which 580 gm consisted of the fat under investigation. No nausea or other unpleasant symptoms were noted during the course of the investigation.

The data on nitrogen excretion indicate that both fats are without deleterious effect on the nitrogen metabolism. The urinary nitrogen is identical on the rapeseed and cottonseed oil diets (8.62 gm) while the fecal nitrogen values are also

TABLE 2  
Summary of the digestibility experiments on human subjects on a natural diet containing rapeseed oil as the principal fat component

SUBJECT	CALORIC INTAKE	NITROGEN/GM/DAY				UREA-TININE	URINE VOL.	FECES GM/DAY		FAT IN GM/DAY				COEFFICIENT OF DIGESTIBILITY
		Food	Urine	Fecal	Balance			Moist	Dry	Food <sup>1</sup>	EXCRETED			
											Neutral fat, fatty acids	Soaps	Total corrected	
		mg	ml											
Brown	2,500	12.94	8.53	0.77	3.64	1255	2295	72.0	13.3	73.0	1.28	0.72	0.00	100.0
Belloni	2,000	10.36	7.84	0.74	1.78	1659	1936	70.0	16.0	58.4	1.87	1.07	0.00	100.0
Keeper	2,000	10.36	8.54	0.31	1.27	1261	2047	29.4	7.8	58.4	1.10	0.84	0.40	99.4
Kingston	2,000	10.36	7.98	0.73	1.65	1666	1466	144.2	15.9	58.4	1.39	1.17	0.00	100.0
Burns	2,000	10.36	8.66	0.76	0.94	1551	1761	65.3	16.6	58.4	1.98	1.09	0.00	100.0
Oliver	2,000	10.36	7.42	0.90	2.02	1184	1583	59.2	17.3	58.4	2.26	1.12	0.00	100.0
Karrer	2,000	10.36	9.26	1.09	0.01	1546	1769	96.7	21.4	58.4	2.96	2.78	1.50	97.4
Butler	2,000	10.36	10.77	0.54	0.05	1865	1922	75.6	20.8	58.4	3.92	3.73	3.53	94.0
Average		10.68	8.62	0.73	1.33	1498	1847	72.8	16.1	60.2	2.10	1.56	0.68	99.0

<sup>1</sup> Estimated intake from quantity added to the food.

TABLE 3  
*Summary of the digestibility experiments on human subjects on a natural diet containing cottonseed (Wesson) oil as the principal fat component*

SUBJECT	CALORIC INTAKE	NITROGEN/GM/DAY				CREATININE	URINE VOL.	FECES GM/DAY		FAT IN GM/DAY			COEFFICIENT OF DIGESTIBILITY	
		Food	Urine	Fecal	Balance			Moist	Dry	Food <sup>1</sup>	EXCRETED			
											Neutral fat, fatty acids	Total corrected		
						mg	ml							
Brown	2,500	12.68	8.48	0.70	3.50	1422	2441	63.9	12.9	73.0	1.00	2.79	1.23	98.4
Belloni	2,000	10.12	7.93	0.93	1.26	1686	1828	81.0	21.7	58.4	3.00	3.06	1.76	97.2
Keeper	2,000	10.12	9.05	0.61	0.46	1517	1954	27.5	13.3	58.4	5.32	2.10	4.78	91.8
Kingston	2,000	10.12	7.83	0.74	1.55	1833	1546	96.9	15.7	58.4	1.47	2.68	1.04	98.2
Burns	2,000	10.12	9.16	0.42	0.54	1737	1869	70.0	19.9	58.4	2.07	3.35	1.48	97.5
Oliver	2,000	10.12	7.79	0.81	2.52	1242	1609	55.7	15.9	58.4	1.41	2.72	0.98	98.3
Karrer	2,000	10.12	9.15	0.91	0.06	1647	1824	82.1	19.6	58.4	1.77	3.90	1.79	96.9
Butler	2,000	10.12	9.55	0.56	0.01	1777	1604	56.4	17.5	58.4	3.82	3.22	3.57	94.0
Average		10.44	8.62	0.71	1.11	1608	1834	66.7	17.1	60.2	2.48	2.98	2.08	96.5

<sup>1</sup> Estimated intake from quantity added to the food.



practically the same (0.73 and 0.71 gm). An equally favorable positive nitrogen balance obtains in tests with both oils (1.33 and 1.11).

#### DISCUSSION

The present tests indicate that rapeseed and cottonseed oil are almost completely digested in man when one includes in the calculation that portion excreted as soap. The figure for rapeseed oil (99.0) is practically identical with the original value of Holmes ('18) of 98.8, where the soap fraction was not considered. It indicates that a species difference does exist between man and the rat with respect to utilization of rapeseed oil.

Although it is recognized that a comparison of digestibility in rat and man is complicated by the different diets employed in the two cases, such a variation does not bring about a difference in the digestibility of cottonseed oil, but only of rapeseed oil. This would seem to preclude the difference in dietary regime as being responsible for the variation in the digestibility of rapeseed oil in rats and man.

The coefficient of digestibility reported here for cottonseed oil (96.5) also agrees well with earlier reports where the soaps were not taken into consideration. Thus, Langworthy and Holmes ('17) give a value of 97.6 for normal man, while Holt et al. ('35) have reported a figure of 96.9 for normal infants.

The differences in utilization between rapeseed and cottonseed oils, while quite small, would seem to be real from a statistical viewpoint. Thus, in no single instance is there any overlapping of the results of individual tests with cottonseed oil and the average value noted in the rapeseed oil tests. This seems to be largely the result of a higher soap excretion in the cottonseed oil tests as compared with the rapeseed oil experiments (2.98 vs. 1.56 gm). Inasmuch as the diet used is relatively high in calcium, it is also possible that the bivalent ion may have a greater depressing effect on the fatty acids of cottonseed oil than it does on the fatty acids of rapeseed oil (Cheng, Morehouse and Deuel, '49).

The present tests have shown that a low fat diet can be used as a basis for such fat studies, and can be made an extremely appetizing one by incorporation of considerable amounts of the fat under study. Such a procedure probably has advantages over the largely artificial diets formerly employed in such studies in that the ration is tolerated over long periods of time and allows studies to be made of the availability of fats when they are incorporated into a normal diet.

#### SUMMARY

1. The digestibility of rapeseed oil has been found to be 99% in normal men, while that of cottonseed oil was found to be 96.7% when the excretion of soap is also considered.

2. While the differences in values between these fats are only slight, they are apparently significant. They result from the somewhat greater excretion of soap in the cottonseed oil tests.

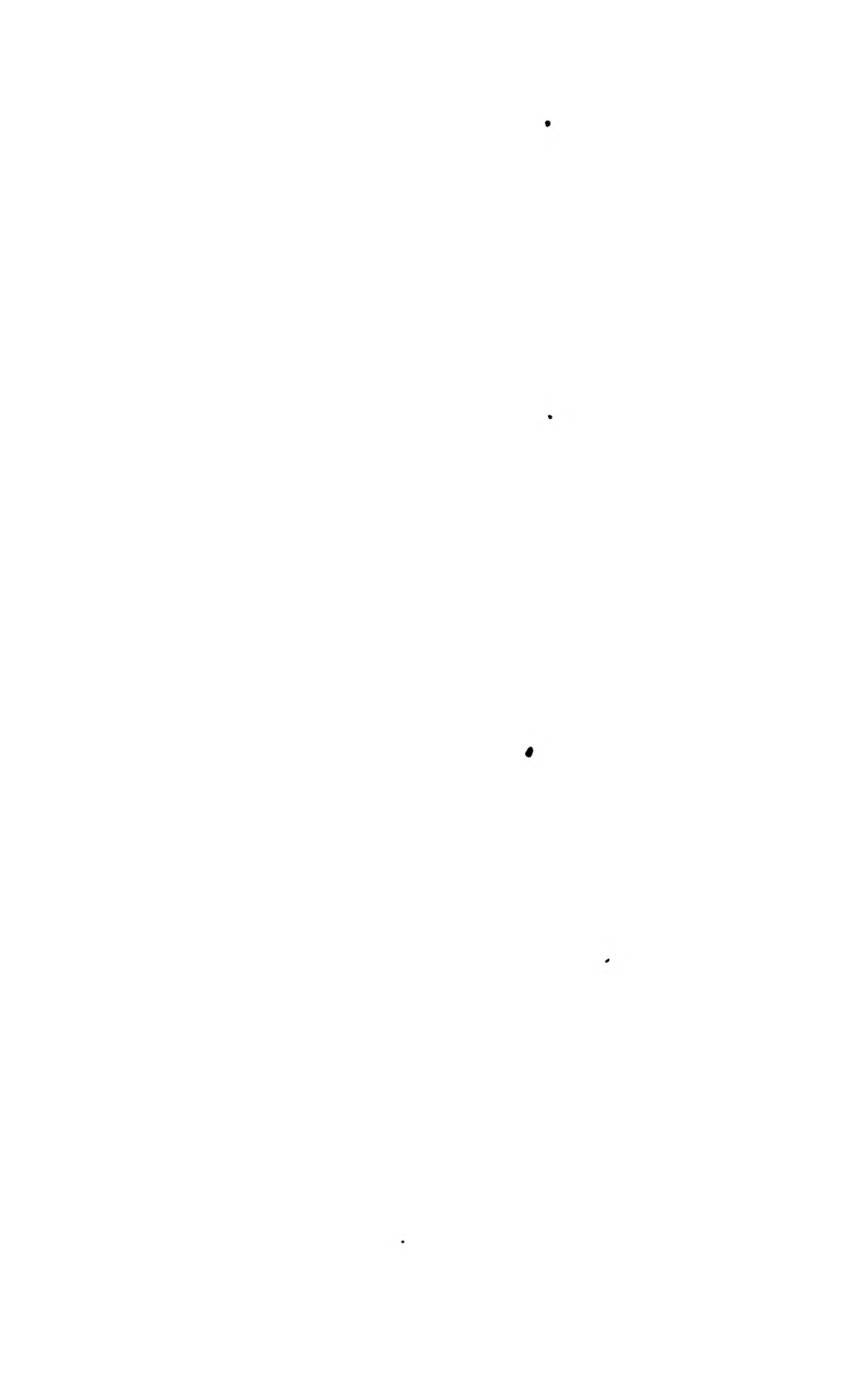
3. The results using rapeseed oil prove that a species difference obtains in respect to digestibility between man and the rat.

4. A basic diet containing a minimum quantity of fat was employed which avoids monotony and provides an appetizing and varied menu.

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## NUTRITIVE PROPERTIES OF PROTEIN IN DIFFERENT CUTS OF BEEF

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In view of the high price of beef, there is need for information concerning the nutritive properties of the meat of one cut as compared with those of another. Since protein is the most important nutritive constituent of lean beef, the question arises as to whether the protein in the less expensive cuts, such as the neck, shank, or chuck, is of as high nutritive value as that in the higher-priced cuts, such as the round, loin or rib. Published results of research on meats do not supply an adequate answer.

The results of earlier investigations suggest that the biological value of the protein in different cuts of beef is materially influenced by the proportion of connective tissue present (Mitchell, '27). Much work has been done by Mitchell and associates ('27, '28) and by others to develop an accurate method for the estimation of collagen in meats, but the results have not been entirely satisfactory. Mitchell et al. ('28) reported that the eye muscle of the rib contained the lowest percentage of collagen, whereas the round, sirloin and porterhouse cuts showed slightly higher proportions. The fore shank contained much more collagen than the rib eye muscle.

The quantitative determination of each of the amino acids essential for the rat, and of cystine, is of fundamental importance in a comparison of the nutritive values of the pro-

tein in different cuts of beef, but the final proof must depend upon the results of animal experimentation. Kraybill ('48) measured the amounts of 10 essential amino acids and cystine in the rib, chuck, neck, plate, rump and flank cuts of both choice and utility grades of beef and reported that significant differences were not found. He suggests that "The methods for estimation of the amino acids may not be sufficiently accurate to determine the small differences existing in these cuts." Kuiken et al. ('48) found that all 10 of the essential amino acids in roast prime ribs of beef are completely available to the rat.

The purpose of the experiments reported in this paper was to determine, by means of feeding tests with young albino rats, the relative growth-promoting values of the protein in the round, loin, rib, chuck, neck and shank cuts from each of two carcasses of beef of different ages. Dried skim milk and mixtures of beef and gelatin were tested for comparative purposes.

## EXPERIMENTAL

### *Products tested*<sup>1</sup>

Cuts from the carcasses of two beef cattle, a young steer and an old cow, were used in the experiments. A brief history of each follows:

(1) A young beef-type Shorthorn steer was weaned at approximately 8 months of age, when it weighed 508 lb. and was graded "low choice" as a feeder. The steer was fed individually for 220 days, the grain mixture consisting of corn 5 parts, oats 3, bran 1, and linseed oil meal 1 part, by weight. Number 2 leafy alfalfa hay was fed as roughage. At the end of the fattening period the steer weighed 905 lb., having made an average daily gain of 1.8 lb. The steer was graded "low choice." The chilled carcass weighed 534

<sup>1</sup> The authors acknowledge, with thanks, the assistance of R. L. Hiner in the slaughter of the animals and the preparation of the cuts for experimental purposes.

lb. and was graded "high good." The fat content and distribution were normal for a carcass of this grade.

(2) A Hereford heifer, beginning at 6 months of age and at a weight of 319 lb., was fed for a period of 10 years a ration low in carotene and vitamin A but otherwise adequate, for the purpose of studying the vitamin A requirements for reproduction. At first sufficient alfalfa leaf meal to furnish 30  $\mu$ g of carotene per kilogram of body weight was fed daily, but at the end of 4 years the carotene was increased to 60  $\mu$ g. The ration consisted of the following ingredients: barley 40%, oats 30%, bran 10%, dried beef pulp 10%, linseed oil meal 4%, soybean meal 4%, iodized salt 1%, and limestone 1%. The ration contained 14.6% protein, 9.1% fiber, 3.2% fat, 0.54% Ca, and 0.47% P. Oat straw was fed as roughage. During a 10-year period the cow gave birth to a number of calves. At the time of slaughter she weighed 1,460 lb. and was in excellent condition. The chilled carcass weighed 940 lb. and was graded "commercial." It was very fat and there was an unusual distribution of intramuscular fat but no evidence of fatty degeneration.

The carcass of each animal was divided into the usual wholesale cuts and the following were selected for experimental purposes: round (rump and shank off), entire loin, rib, chuck, neck, and combined fore and hind shanks. The lean meat from each cut was cut into small pieces, frozen at 0°F., ground and dehydrated at 120°F. Considerable difficulty was experienced in the dehydration of the lean meat of certain cuts from the Hereford cow because of the unusual deposits of intramuscular fat. The dehydrated beef was thoroughly extracted with ethyl ether, ground fine and stored in friction-top tin cans at approximately 0°F.

Dried skim milk of a well-known brand, packed in sealed tin cans, was also tested.

Beef sample 4533, used in an experiment with gelatin, was a mixture of equal proportions of dehydrated chuck, round and loin from the Hereford cow.

*Diets fed*

The dehydrated beef from each cut from each carcass and dried skim milk were incorporated in diets in such proportions as to supply 1.2, 1.6 and 2% of nitrogen corresponding to 7.5, 10 and 12.5% protein. In addition, diets containing 1.2% nitrogen from beef were supplemented with 0.25% L-cystine, since Hoagland et al. ('48) had previously found beef protein to be deficient in this amino acid. Diets containing 1.6% nitrogen were also prepared in which 10, 15 or 20% of the total nitrogen was supplied as gelatin and the remainder as dehydrated beef.

The following quantities of B vitamins were added to 100 gm of each diet: thiamine hydrochloride 0.3 mg, riboflavin 0.3 mg, pyridoxine hydrochloride 0.6 mg, calcium pantothenate 1.5 mg, and choline chloride 80 mg.

The fat-soluble vitamins were dissolved in lard and added in such quantity that 1 gm of the diet would contain 5 I. U. of vitamin A, one unit of vitamin D and 20  $\mu$ g of alphatocopherol. Sufficient kettle-rendered lard was added to make 10% fat in the diet. Salt mixture (Hoagland and Snider, '40) amounting to 4% and sufficient dextrin to make up 100% completed the diet. Each diet was made up in the quantity of 1 kg at one time and was stored in one-quart glass jars in a refrigerator at 40°F. during the course of the experiments.

The feeding tests were conducted in the manner previously described by the present writers ('47).

## RESULTS

*Experiment 1*

The average results of the feeding tests with groups of 8 young male albino rats to determine the relative growth-promoting values of the protein in 6 cuts of beef from the carcass of a Shorthorn steer are shown in table 1. The gain in weight per gram of nitrogen consumed was selected as the most reliable basis for comparisons in this and the following experiments.



TABLE 1

*Nutritive value of the protein in different cuts of beef from the carcass of a Shorthorn steer in 30-day tests with groups of 8 male albino rats*

no.	DESCRIPTION OF DIETS Cut of beef	PRO- TEIN CON- TENT	NITRO- GEN CON- TENT	GAIN IN WEIGHT	FEED CON- SUMED	FEED CON- SUMED PER GRAM GAIN IN WEIGHT	GAIN IN WT. PER GRAM OF NITRO- GEN
		%	%	gm	gm	gm	gm
Experiment 1							
1	Shank	7.5	1.2	85	323	3.80	21.9
2	Round	7.5	1.2	75	282	3.76	22.1
3	Loin	7.5	1.2	76	290	3.82	21.7
4	Neck	7.5	1.2	78	300	3.85	21.6
5	Rib	7.5	1.2	72	284	3.94	20.9
6	Chuck	7.5	1.2	81	309	3.81	21.7
	Average	7.5	1.2	78	298	3.83	21.7
7	Shank	10.0	1.6	118	344	2.92	21.4
8 <sup>1</sup>	Round	10.0	1.6	115	332	2.88	21.7
9	Loin	10.0	1.6	125	356	2.85	21.8
10	Neck	10.0	1.6	121	350	2.89	21.6
11	Rib	10.0	1.6	120	348	2.90	21.5
12	Chuck	10.0	1.6	126	360	2.86	21.9
	Average	10.0	1.6	121	348	2.88	21.7
13	Shank	12.5	2.0	151	374	2.48	20.1
14	Round	12.5	2.0	157	383	2.44	20.5
15	Loin	12.5	2.0	148	374	2.53	19.8
16	Neck	12.5	2.0	151	386	2.56	19.5
17	Rib	12.5	2.0	142	368	2.59	19.1
18	Chuck	12.5	2.0	158	390	2.47	20.3
	Average	12.5	2.0	151	379	2.51	19.9
19	Shank + 0.25% cystine	7.5	1.23	109	340	3.12	26.0
20	Round + 0.25% cystine	7.5	1.23	114	340	2.73	27.3
21	Loin + 0.25% cystine	7.5	1.23	117	345	2.95	27.5
22	Neck + 0.25% cystine	7.5	1.23	113	351	3.11	26.2
23	Chuck + 0.25% cystine	7.5	1.23	121	366	3.02	26.8
	Average	7.5	1.23	115	348	2.99	26.8

<sup>1</sup> Seven rats.

A statistical analysis of the experimental data was made by means of Fisher's "t" values for the significance of the difference between the means of the gain in weight per gram of nitrogen consumed. The following probability values were obtained:

- (1) 1.2% nitrogen, diet 5 vs. diets 1, 2, 3, 4, and 6—not significant
- (2) 1.6% nitrogen, diet 7 vs. diets 8, 9, 10, 11, and 12—not significant
- (3) 2.0% nitrogen, diet 17 vs. diets 13, 14, and 18—significant ( $<0.02$ , 0.001, 0.01); diet 16 vs. diets 13, 14, and 18—significant ( $<0.05$ , 0.001, and 0.02)
- (4) 1.2% nitrogen + 0.25% cystine, diet 19 vs. diets 20, 21, 22, and 23—not significant
- (5) 1.2% nitrogen vs. 1.2% nitrogen + 0.25% cystine, diet 1 vs. 19, 2 vs. 20, 3 vs. 21, 4 vs. 22, and 6 vs. 23—all significant ( $<0.001$ )

The results of the tests with diets 1 to 6 (table 1) indicate clearly that there were no significant differences between the growth-promoting values for the protein in the different cuts of beef when the diets contained 1.2% nitrogen.

When the diets contained 1.6% nitrogen, the results of the tests with diets 7 to 12 (table 1) likewise show no significant differences between the growth-promoting values for the different cuts of beef.

When the diets contained 2.0% nitrogen, the growth-promoting values for the protein in the neck and rib (diets 16 and 17) were significantly lower than the values for the shank, round and chuck (diets 13, 14 and 18). The average growth-promoting value of the three less expensive cuts—the shank, neck and chuck—was 20.0, as compared with 19.8 for the three more expensive cuts, the round, loin and rib.

The addition of 0.25% cystine to diets 19 to 23, containing 1.2% protein nitrogen, caused a marked increase in the rate of growth and efficiency of protein utilization as compared with diets 1 to 6, which did not contain added cystine. There were no significant differences between the growth-promoting values of the protein in any of the cuts of beef when supplemented with cystine. The rib cut of beef was not tested because of an inadequate supply of meat.

The results of the experiments with the 6 cuts of beef from the Shorthorn steer show that there were no significant differences between the growth-promoting values of the protein in the different cuts when the diets contained 1.2 or 1.6% nitrogen, or 1.2% protein nitrogen + 0.25% cystine. Only when the diets contained 2.0% nitrogen was a significant difference found between the protein values for the rib and neck and three other cuts. The reason for these differences is not apparent. On the whole, the results of this experiment indicate that the protein in the less expensive cuts, the shank, neck and chuck, was of as high nutritive value as the protein in the more expensive cuts, the round, loin and rib.

It is noteworthy that the average growth-promoting value of the protein in all cuts of beef was identical at the 1.2 and 1.6% levels of nitrogen intake, and the value at the 2.0% nitrogen level was only slightly lower. On the other hand, there was a marked increase in the rate of growth of the rats as the percentage of protein was increased.

### *Experiment 2*

This experiment was a duplicate of experiment 1 except that the cuts of beef were obtained from the carcass of a Hereford cow that had been fed a ration low in carotene for a long period of time. The results are shown in table 2.

A statistical analysis was made of the experimental data by the procedure followed in the preceding experiment, with the following results:

- (1) 1.2% nitrogen, diet 2A vs. 3A, 4A, 5A, and 6A—significant ( $<0.05$ , 0.03, 0.05, 0.01). 2A vs. 1A, not significant ( $<0.1$ )
- (2) 1.6% nitrogen, differences not significant
- (3) 2.0% nitrogen, diet 17A vs. 18A—significant ( $<0.02$ ); other differences not significant
- (4) 1.2% nitrogen + 0.25% cystine, diet 23A vs. 19A, 20A, 21A, and 24A—significant ( $<0.05$ , 0.05, 0.01, 0.01); other differences not significant
- (5) 1.2% nitrogen vs. 1.2% nitrogen + 0.25% cystine, diets 1A vs. 19A, 2A vs. 20A, 3A vs. 21A, 4A vs. 22A, 5A vs. 23A, 6A vs. 24A—all significant ( $<0.001$ )

TABLE 2

*Nutritive value of the protein in different cuts of beef from the carcass of a Hereford cow in 30-day tests with groups of 8 male albino rats*

DESCRIPTION OF DIETS		PRO- TEIN CON- TENT	NITRO- GEN CON- TENT	GAIN IN WEIGHT	FEED CON- SUMED	FEED CON- SUMED PER GRAM GAIN IN WEIGHT	GAIN IN WT. PER GRAM OF NITRO- GEN
no.	Cut of beef						
		%	%	gm	gm	gm	gm
		Experiment 2					
1A	Shank	7.5	1.2	67	263	3.93	21.1
2A	Round	7.5	1.2	74	276	3.73	22.2
3A	Loin	7.5	1.2	69	278	4.03	20.4
4A	Neck	7.5	1.2	67	272	4.06	20.4
5A	Rib	7.5	1.2	71	279	3.93	21.1
6A	Chuck	7.5	1.2	70	289	4.13	20.1
	Average	7.5	1.2	70	276	3.97	20.9
7A	Shank	10.0	1.6	97	295	3.04	20.6
8A	Round	10.0	1.6	100	315	3.15	19.9
9A	Loin	10.0	1.6	100	316	3.16	19.7
10A	Neck	10.0	1.6	104	307	2.95	21.1
11A	Rib	10.0	1.6	100	302	3.02	20.6
12A	Chuck	10.0	1.6	98	316	3.22	19.3
	Average	10.0	1.6	100	309	3.09	20.2
13A	Shank	12.5	2.0	131	342	2.61	19.2
14A	Round	12.5	2.0	143	355	2.48	20.1
15A	Loin	12.5	2.0	141	357	2.53	19.7
16A	Neck	12.5	2.0	135	351	2.60	19.3
17A	Rib	12.5	2.0	125	329	2.63	18.9
18A	Chuck	12.5	2.0	139	347	2.50	20.0
	Average	12.5	2.0	136	347	2.56	19.5
19A	Shank + 0.25% cystine	7.5	1.23	109	331	3.04	26.7
20A	Round + 0.25% cystine	7.5	1.23	104	316	3.04	26.6
21A	Loin + 0.25% cystine	7.5	1.23	102	322	3.16	25.8
22A	Neck + 0.25% cystine	7.5	1.23	114	345	3.03	26.8
23A	Rib + 0.25% cystine	7.5	1.23	119	344	2.89	28.0
24A	Chuck + 0.25% cystine	7.5	1.23	88	284	3.23	25.2
	Average	7.5	1.23	106	324	3.07	26.5

The results of the experiment with the diets containing 1.2% nitrogen and using the different cuts from the carcass of the Hereford cow suggest that the protein in the round was of higher nutritive value than that in the other cuts, except the shank, but there were no other significant differences. The fact that the protein in the round was not superior to that in the other cuts at the higher levels of intake indicates that the apparent superiority of the round at the 1.2% level of nitrogen intake was fortuitous.

When the diets contained 1.6% nitrogen there were no significant differences between the growth-promoting values of the protein in the different cuts of beef.

The results of the tests with the diets containing 2% nitrogen indicate that the protein in the chuck (diet 18A) was of significantly higher nutritive value than that in the rib (diet 17A). Other differences were not significant.

When the diets contained 1.2% nitrogen + 0.25% cystine there was a marked increase in the rate of growth and in the efficiency of utilization of the protein in each cut of beef as compared with the diets containing 1.2% nitrogen but no added cystine. The protein in diet 23A (rib + cystine) had an unusually high growth-promoting value, significantly higher than that for any other diet except 22A (neck + cystine).

In this experiment there were only relatively small differences between the average growth-promoting values for all cuts at the 7.5, 10, and 12.5% levels of protein intake. The average growth value for the protein at each level of intake was slightly higher for the cuts from the Shorthorn steer (table 1) than for those from the Hereford cow (table 2) but a statistical analysis of the experimental data indicates that the differences were not significant.

### *Experiment 3*

This experiment was conducted to determine (1) the growth-promoting value of the protein in dried skim milk at the 1.2, 1.6, and 2.0% levels of nitrogen intake, and (2)

the effects of the addition to beef of different proportions of gelatin on the nutritive value of the protein at the 1.6% level of nitrogen intake. In order to simplify comparisons between diets in this and previous experiments, the factor 6.25 was used to convert nitrogen to protein. The results are shown in table 3.

TABLE 3

*Nutritive value of the protein in dried skim milk and in mixtures of dried raw beef and gelatin in 30-day tests with groups of 8 male albino rats*

DESCRIPTION OF DIETS		PRO- TEIN <sup>1</sup> CON- TENT	NITRO- GEN CON- TENT	GAIN IN WEIGHT	FEED CON- SUMED	FEED CON- SUMED PER GRAM GAIN IN WEIGHT	GAIN IN WT. PER GRAM OF NITRO- GEN
no.	Source of protein						
Experiment 3							
		%	%	gm.	gm.	gm.	gm.
1B	Dried skim milk	7.5	1.2	88	328	3.73	22.2
2B	Dried skim milk	10.0	1.6	124	375	3.02	20.6
3B	Dried skim milk	12.5	2.0	132	362	2.74	18.2
4B	Dried beef 4533	10.0	1.6	124	367	2.96	21.0
5B	Dried beef 4533 + 10% gelatin nitrogen	10.0	1.6	107	348	3.25	19.2
6B	Dried beef 4533 + 15% gelatin nitrogen	10.0	1.6	109	352	3.23	19.3
7B	Dried beef 4533 + 20% gelatin nitrogen	10.0	1.6	97	337	3.47	17.9

<sup>1</sup> Protein content =  $N \times 6.25$ .

A statistical analysis was made of the experimental data with the following results:

- (1) Diet 1B vs. 2B and 3B—significant ( $<0.01$ , 0.001)
- (2) Diet 2B vs. 3B—significant ( $<0.02$ )
- (3) Diet 1B vs. average of diets 1 to 6—not significant
- (4) Diet 1B vs. average of diets 1A to 6A—significant ( $<0.02$ )
- (5) Diet 2B vs. average of diets 7 to 12—significant ( $<0.01$ )
- (6) Diet 2B vs. average of diets 7A to 12A—not significant
- (7) Diet 3B vs. average of diets 13 to 18—significant ( $<0.001$ )
- (8) Diet 3B vs. average of diets 13A to 18A—significant (0.001)
- (9) Diet 4B vs. 5B, 6B, 7B—significant ( $<0.001$ , 0.01, 0.001)
- (10) Diet 5B vs. 7B—significant ( $<0.01$ )
- (11) Diet 6B vs. 7B—significant ( $<0.05$ )

The results of the experiments with dried skim milk show an increase in the rate of growth of the rats but a decrease in the efficiency of utilization of the protein as the percentage of nitrogen in the diets was increased from 1.2 to 1.6 or 2.0%. A comparison of the growth-promoting value of milk protein (gain per gram of nitrogen consumed) at each level of intake (table 3) with the average values for all cuts of beef at the same level of intake (tables 1 and 2) yields the following results: (1) At the 1.2% nitrogen level, the protein in dried skim milk had approximately the same growth value as all cuts of beef from the Shorthorn steer (table 1), but one significantly higher than the average value for all cuts from the Hereford cow (table 2); (2) At the 1.6% nitrogen level the average value for all cuts from the Shorthorn steer (table 1) was significantly higher than the value for milk, but the average value of the cuts from the Hereford cow (table 2) was approximately the same as that for milk; (3) At the 2.0% nitrogen level, the average value for all the cuts from each carcass of beef was significantly higher than the value for milk.

The admixture of different proportions of gelatin with dehydrated raw beef materially lowered the growth-promoting values of the mixtures as compared with those of beef alone. When nitrogen constituted 1.6% of the diet, the replacement of 10, 15 or 20% of the beef nitrogen by gelatin nitrogen resulted in a marked decrease in the rate of growth and in the efficiency of protein utilization. The fact that the protein in the less expensive cuts of beef, such as the neck, shank and chuck, did not differ consistently in growth-promoting value from the protein in the more expensive cuts such as the round, loin and rib, suggests that the difference in collagen content of the two groups of cuts is not sufficient materially to affect the nutritive value of the total protein in the meat.

#### SUMMARY

The relative nutritive or growth-promoting values of the protein in 6 different cuts of beef from each of two car-

casses, one of a Shorthorn steer graded "high good" and the other a Hereford cow graded "commercial," were determined by feeding tests with young male albino rats. The following cuts of beef were tested: round, loin, rib, chuck, neck, and combined fore and hind shanks.

When protein constituted 7.5% of the diets, there were no significant differences between the nutritive values of the protein in the different cuts from the Shorthorn steer. In tests with the cuts from the Hereford cow, the protein in the round had a higher value than that in the other cuts except the shanks.

When protein constituted 10% of the diets, there were no significant differences between the nutritive values of the protein in the different cuts of beef, either from the Shorthorn steer or from the Hereford cow.

When the diets contained 12.5% protein, the growth-promoting values of the protein in the rib and neck from the Shorthorn steer were slightly lower than the values for the shank, round or chuck. The protein in the rib from the Hereford cow had a slightly lower value than the protein in the chuck.

The average growth-promoting value of the protein at each level of intake was slightly higher for all the cuts from the Shorthorn steer than for those from the Hereford cow, but the differences were not statistically significant.

When diets containing 7.5% protein were supplemented with 0.25% cystine, there was a marked increase in the rate of growth and in the efficiency of utilization of the protein in each cut of beef from each animal. There were no significant differences between the nutritive values of the protein in the different cuts from the Shorthorn steer. The protein in the rib from the Hereford cow had a significantly higher value than that in any other cut, except the neck.

The average nutritive value of the protein in the cuts of beef from the Shorthorn steer, at the 7.5% level of protein intake, was approximately the same as the value for dried skim milk. When the diets contained 10.0 or 12.5% protein,



the beef protein was of higher nutritive value than the milk protein.

The average nutritive value of the protein in the cuts from the Hereford cow was lower than the value for milk at the 7.5% protein level, the same at the 10% level, but higher at the 12.5% level.

The admixture of gelatin with beef, in the proportions of 10, 15, or 20% of the total nitrogen in the diets, definitely lowered the nutritive value of the total protein when the diets contained 1.6% nitrogen.

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# PROCEEDINGS OF THE THIRTEENTH ANNUAL MEETING OF THE AMERICAN INSTITUTE OF NUTRITION

THE STATLER HOTEL AND THE MASONIC TEMPLE  
DETROIT, MICHIGAN, APRIL 18-22, 1949

## COUNCIL MEETINGS

Council meetings were held in the Statler on Sunday and Monday, April 17 and 18. Formal actions of the Council are reported in the minutes of the business meetings.

## SCIENTIFIC SESSIONS

The scientific program consisted of 8 half-day sessions of papers grouped according to subject matter and a symposium on "Nutrition in Preventive Medicine" held on the evening of April 21. Ninety-four papers were presented at the scientific sessions and 4 papers were read by title. The Institute also participated in the Joint Session of the Federation on the evening of April 19.

## BUSINESS SESSIONS

Two business meetings were held: one at 4:30 P.M., Tuesday, April 19, and one at 4:30 P.M., Thursday, April 21. President E. M. Nelson presided at the first meeting and Vice-President C. G. King was in charge of the second meeting. Items of business received attention as follows:

*Tuesday, April 19, 4:30 P.M.* The meeting was called to order by President E. M. Nelson. The President appointed Dr. H. R. Bird and Dr. A. Arnold to serve as a Tellers' Committee and ballots on the election of officers were transmitted to this Committee by the Secretary.

President Nelson referred to the Proceedings of last year's meeting, as published in the June, 1948, issue of *THE JOURNAL OF NUTRITION*, and a call was made for corrections or additions. No objections being noted, the published transactions of last year's meeting were declared approved.

The President reported that since the last meeting the Special Committee charged with the responsibility of setting up the rules and regulations for the award made available by the Nutrition Foundation, Inc., had recommended that this award be called the "Osborne and Mendel Award." This recommendation had been approved by the Council.

The report of the Treasurer, N. R. Ellis, was submitted. The Auditing Committee (Dr. Elsa Orent-Keiles and Dr. Esther L. Batchelder) presented a report stating that the books of the Treasurer were in order. The Treasurer's report was adopted.

The Secretary reported that the Executive Committee of the Federation had taken the following actions:

Decided that the next annual meeting would be held at Atlantic City, New Jersey, during the week beginning April 9.

Recommended that the Federation assessment be set at three dollars per member.

Recommended that the secretaries of the constituent societies serve as the Editorial Board for the Federation Proceedings, replacing the Control Board which has been functioning in this capacity.

Dr. H. J. Deuel, Jr., reported that Dr. George R. Cowgill had been elected by the Editorial Board of *THE JOURNAL OF NUTRITION* to serve as Editor for the coming 5-year period, and that Dr. Cowgill had accepted. Dr. Deuel read the following motion which had been unanimously adopted by the Editorial Board:

"It was moved that the Editor be cordially thanked for his services during the past 10 years, that he be re-elected for the ensuing 5 years, and that he be urgently requested to accept the action of the Board."

The annual report of the Editor of THE JOURNAL OF NUTRITION was submitted by Dr. G. R. Cowgill. The report follows:

Two volumes (Nos. 35 and 36) were published during 1948; they contained 64 and 67 articles, respectively, which was an increase of 10 papers over the number published in 1947. The number of pages per article proved to be almost identical with that for the previous year.

During 1948, because of rising publication costs, Wistar Institute found it necessary to review every aspect of its printing shop operations. Following its study, the subscription price for most of the Wistar publications was increased and certain other actions taken. With respect to THE JOURNAL OF NUTRITION (a) the subscription rate to non-society members was raised from \$5.00 to \$7.00 per volume beginning in January, 1949; (b) the number of volumes to be published annually was increased from two to three; (c) the number of pages per volume was reduced from 720 to 600, the net effect of which is the publication of at least 1800 instead of 1440 pages per year; and finally, (d) the Editor was allowed additional pages not to exceed 200 annually as he might need them in order to secure the most prompt publication of acceptable material. The Editor proposed to Wistar Institute — and it was accepted — that there be no change in the subscription rate for members of the American Institute of Nutrition, who are required to subscribe as a condition of membership. Inasmuch as (a) members of the society had already paid through annual dues for two volumes of the Journal and thus were entitled to issues up to and including that for April, 1949, and (b) new dues would have to be voted at the next annual meeting scheduled for Detroit in April, 1949, the Editor further proposed to Wistar Institute — and it was accepted — that any change in the annual cost of the Journal to society members not be effective until after the next annual meeting, at which annual dues would be discussed and then voted. If the new arrangement is adopted, the part of the annual dues

covering subscription to the JOURNAL (for three volumes) will be \$7.50.

A cumulative index covering a 10-year period (1938-1948) and 21 volumes (16-36 inclusive) is being prepared. It should be ready during the summer of 1949.

A motion was passed setting the total dues of members at \$11.50 for the next year. Of this amount, \$1.00 is allocated to the Institute of Nutrition, \$3.00 to the Federation and \$7.50 for subscription to THE JOURNAL OF NUTRITION.

Dr. G. R. Cowgill discussed in further detail the preparation of the 10-year cumulative index. He presented the request of the Wistar Institute of Anatomy and Biology that the American Institute of Nutrition assume financial responsibility for publishing the index to the extent of the cost of one volume per member. Dr. J. R. Murlin moved that, if a deficit occurs in publishing the index, the Institute of Nutrition would assume responsibility equal to the cost of one copy per member, less a credit for the number of copies that have been purchased by members. The motion was passed.

A motion was passed authorizing the Secretary to prepare and distribute with the ballots for election of officers a printed directory of members with their affiliations.

The report of the Committee on Pathological Registries, consisting of Dr. R. E. Johnson, Chairman, Dr. O. A. Bessey and Dr. W. H. Sebrell, on the desirability and feasibility of sponsoring a Registry of Nutritional Pathology as a new unit of the American Registry of Pathology was presented by Dr. Johnson. The Committee recommended that such a registry be sponsored by the American Institute of Nutrition and submitted an organization plan by which this registry would be set up. The report was adopted.

President Nelson requested authority to appoint a committee to study present procedures used in selecting recipients of awards and in conferring these awards and to make recommendations at the next annual meeting that will lead to improved procedures. The request was approved.

The Tellers' Committee reported the election of the following officers for the year beginning July 1, 1949:

*President*  
CHARLES G. KING  
*Vice President*  
WENDELL H. GRIFFITH  
*Councillor*  
ARTHUR H. SMITH

*Associate Editors*  
WILLIAM J. DARBY  
DAVID M. HEGSTED  
PEARL P. SWANSON

The meeting was adjourned at 5:20 P.M.

*Thursday, April 21, 4:30 P.M.* The second business meeting was called to order by Vice President C. G. King. The Tellers' Committee reported the results of the voting upon suggestions for the nominating committee. The names of the 10 members receiving the highest number of votes were submitted for the guidance of the President.

The Secretary presented the report of the Committee on Nomenclature (C. A. Elvehjem, Chairman; E. M. Nelson, A. D. Welsh, H. J. Almquist). The report was adopted. This report is as follows:

"It is recommended that the name folic acid and the synonym folacin be adopted for the compound which has been identified as N-[4- { [(2-amino-4-hydroxy-6-pteridyl) methyl] amino } benzoyl] glutamic acid and which has previously been designated by several names including pteroylglutamic acid, vitamin B<sub>9</sub> and *L. casei* factor.

It is recommended that the name pyridoxal be adopted for the compound which has been identified as 2-methyl-3-hydroxy-4-formyl-5-hydroxy-methylpyridine.

It is recommended that the name pyridoxamine be adopted for the compound which has been identified as 2-methyl-3-hydroxy-4-aminomethyl-5-hydroxymethylpyridine.

It is further recommended that the term vitamin B<sub>6</sub> be used as a group name to include pyridoxal, pyridoxamine and pyridoxine, and that these specific names be used only where the corresponding chemical compound is meant. (This recommendation specifically recognizes that the terms 'vitamin B<sub>6</sub>' and 'pyridoxine' should no longer be used synonymously.)"

The Secretary presented a resolution submitted by the Executive Committee of the Federation concerning policies used in selecting and developing the Medical Service Corps, Medical Allied Sciences Section, U.S. Army. A motion was passed adopting the resolution and recommending that it be transmitted to Surgeon General R. W. Bliss. The resolution is as follows:

"The Executive Committee of the Federation of American Societies for Experimental Biology recommends to the respective member societies that they transmit to the office of the Surgeon General, United States Army, attention, Major General R. W. Bliss, a resolution of the following nature:

WHEREAS, the members of our organization are greatly interested in the development of policies that will lead to maximum military effectiveness of scientific personnel,

Be it resolved that we respectfully urge further and continued consideration of the recommendations formulated at the Medical Service Corps Conference, Medical Allied Sciences Section, May 27 and 28, 1948; and

Be it further resolved that in order to keep our members informed concerning their opportunities and responsibilities, similar conferences should be scheduled to review progress and to discuss the related problems of mutual interest."

The report of the Council on the election of new members was adopted.

The following persons were elected to membership in the American Institute of Nutrition:

James B. Allison  
A. E. Axelrod  
Elizabeth C. Callison  
Dena C. Cederquist  
Charles S. Davidson  
Paul F. Fenton  
Gottfried S. Fraenkel  
Charles R. Grau  
Max K. Horwitt  
H. C. Hou  
Carl J. Koehn

Frank H. Kratzer  
Willard A. Krehl  
Henry A. Lardy  
Frieda L. Meyer  
Walther H. Ott  
Leo T. Samuels  
Nevin S. Scrimshaw  
William C. Sherman  
Mary Speirs  
Carleton R. Treadwell  
Le Roy Voris



Discussion of the procedures by which awards are made brought out the points that a re-examination of the present procedures is in order and that efforts should be made to obtain greater stimulation and more publicity in regard to the awards made through the Institute of Nutrition.

There was considerable discussion concerning the desirability of presenting a better scientific program. Dr. E. W. McHenry presented a motion to have the President appoint a committee of 5 members to study present procedures regarding the program of the annual meeting and to make recommendations to the Program Committee and to the Institute at its next annual meeting of ways by which the scientific program may be improved. The motion was passed.

The meeting was adjourned at 5:20 P.M.

#### DINNER AND PRESENTATION OF AWARDS

The Institute held its annual dinner on Wednesday evening, April 20, 1949, in the Book-Cadillac Hotel.

Very stimulating addresses were made by President E. M. Nelson, Dr. W. C. Russell and Dr. C. G. King. Dr. Nelson spoke upon his work as a delegate to a meeting in London, June 10 and 11, 1948, of the Provisional Committee to consider the formation of an International Union of Nutrition Societies. At this conference it was agreed that the name of the proposed organization should be the "International Union of Nutrition Sciences." A tentative constitution and by-laws for the proposed union was prepared and adopted. Application has been made to the International Congress of Scientific Unions for the establishment of an International Union of Nutrition Sciences and this application will be acted upon in September, 1949. Dr. Russell spoke upon the activities of the National Research Council, with special reference to the work of the Food and Nutrition Board. Dr. King discussed the operation of the Medical Allied Services Section of the Medical Corps of the Army, particularly as it relates to the development of scientific information and the opportunities for a scientific career in the Army.

The Mead Johnson Company Award was divided equally between two recipients, Dr. Karl A. Folkers and Dr. Mary S. Shorb. The award was granted to Dr. Folkers for "isolating in crystalline form a new chemical substance, designated as vitamin B<sub>12</sub>, that is active in the treatment of pernicious anemia and of importance in other phases of nutrition," and to Dr. Shorb for "pointing out the possible connection between the anti-anemia factor and a growth factor for bacteria and for developing a bacteriological assay procedure that materially assisted in the isolation of the compound designated as vitamin B<sub>12</sub>."

The Borden Award in Nutrition was presented to Dr. Harry J. Deuel, Jr., for "research emphasizing the nutritive importance of components of dairy products."

The Osborne and Mendel Award was presented to Dr. William C. Rose for "his contributions to our knowledge of nitrogen metabolism and the amino acid requirements of experimental animals and man."

#### COMMITTEES FOR 1949-1950

President E. M. Nelson appointed the following committees for the year beginning July 1, 1949:

##### *Nominating Committee*

ICIE MACY HOOBLER, *Chairman*

E. W. CRAMPTON

H. J. DEUEL, JR.

L. A. MAYNARD

F. J. STARE

##### *Committee on Rules for Awards*

W. C. RUSSELL, *Chairman*

H. E. CARTER

R. R. SEALOCK

C. D. TOLLE

J. WADDELL

*Committee on Program Policy*

W. H. GRIFFITH, *Chairman*

G. R. COWGILL

F. S. DAFT

R. M. LEVERTON

E. W. MCHENRY

*Committee on Registry of Nutritional Pathology*

R. E. JOHNSON, *Chairman*

O. A. BESSEY

P. L. PHILLIPS

W. H. SEBRELL

*Institute Representatives on Joint Committee on Nomenclature*

H. J. ALMQUIST

C. A. ELVEHJEM

Respectfully submitted,

JOSEPH H. ROE, *Secretary*

American Institute of Nutrition



# RELATIVE TO EGG BIOLOGICAL VALUES AND REPLACEMENT VALUES OF SOME CEREAL PROTEINS IN HUMAN SUBJECTS <sup>1</sup>

LELAND C. CLARK, HELEN RYER AND JOHN R. MURLIN  
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(Received for publication December 26, 1948)

This study on a group of 11 young men was performed in August and September, 1943. Four cereal breakfast foods — two of them whole oat products, one three-quarters oats, and another whole wheat — were tested on these subjects alternately with a whole egg diet, and the biological and replacement values of the contained protein calculated from nitrogen balances against similar balances from whole egg protein.

The whole oat proteins will be designated no. 1 and no. 2. The first <sup>2</sup> is sold in 5-lb. paper bags to institutions, like hospitals, hotels and restaurants, the second <sup>3</sup> in 1-lb. packages through the retail trade. The third product,<sup>4</sup> which was new in 1943, consisted of "75% ground oatmeal, 20% corn and rye flours, and the remaining 5% made up of salt, sugar, sodium phosphate, calcium carbonate, vegetable oil, iron salts, niacin, thiamin and riboflavin." We understand that in the course of its preparation the mixture is cooked to softness and squeezed through presses to make little doughnut-shaped rings, which are then dried and puffed. This latter process is carried out by subjecting the product "to a pressure of

<sup>1</sup> This work was supported by a grant from the Quaker Oats Company of Chicago.

<sup>2</sup> "Buckeye" Rolled Oats, made by the Quaker Oats Company.

<sup>3</sup> "H-O," manufactured by the Hecker Products Company.

<sup>4</sup> "Cheerios," now called "Cheerios," manufactured by General Mills, Inc.

80 to 100 lbs. at a temperature of 190°C. to 232°C. for 5½ to 6½ minutes," then letting off the pressure to produce the effect of popping corn. The starch granules are disrupted and are thus more easily attacked by the digestive juices. This product is here called the "puffed mixed cereal."

The 4th product,<sup>5</sup> made from whole wheat, is processed as follows: "It is 'scoured' to remove beard, fine dust and loose particles of the outer bran, then is steam cooked to 118°C., after addition of cane sugar (7%), salt (3%), and malt syrup (0.5%)." It is then dried and flaked (hence it is here called "flaked wheat") by passage between warm rollers and finally toasted "in hot-air ovens where the temperature of the hot air is varied along the line but at no point exceeding 180°C." The temperature of the product is, of course, less than that of the hot air. This last-named cereal was used in a former study (Murlin, Nasset and Marsh, '38) and a considerable reduction in the biological value of the protein as compared with that of other whole wheat products was obtained. We were interested to see if the values obtained 6 years earlier could be duplicated, or whether some improvement had been made to conserve the biological value of the protein. Stewart, Hensley and Peters ('43) have shown that the capacity to maintain growth in rats is definitely impaired by subjecting a mixture of oats, corn and rye (presumably identical with the product we have called "puffed mixed cereal") to the explosion or puffing process, as compared with the growth obtained on a similar mixture of the same cereals not so treated.

#### METHOD

The egg replacement method originally described by Sumner, Pierce and Murlin ('38) was adopted because it provides a diet which can be tolerated much longer and with far less alimentary distress by human subjects than the no-protein control diet employed in the more rigid biological value method of Thomas ('09) and of Martin and Robison ('24). A biological value of equal validity for comparative purposes

<sup>5</sup>"Wheaties," also manufactured by General Mills, Inc.

with that of these authors can be calculated on the assumption that the egg standard diet is attended by no greater alimentary losses of nitrogen than an extremely low protein diet,<sup>6</sup> or one containing no protein at all.

As in previous work from this laboratory, the egg standard diet and the experimental test diet each contained approximately 80% of the protein from egg or cereal, 10% from "4X" cream and butter, and 10% from all the remaining constituents: viz., a plain salad of lettuce and ripe tomato with seeds removed, a dish of applesauce of standard make, and orange marmalade or jam. The distribution of calories among the three proximate principles averaged 5% for protein, 50.1% for carbohydrate and 44.9% for fat. The ranges were from 4.5 to 5.4% for protein, 48.9 to 51.7% for carbohydrate, and 43.0 to 46.1% for fat (table 1). Calories per kilogram body weight per day averaged 45 and per square meter body surface (DuBois) 1,720 per day.

To make sure that each subject received sufficient vitamins throughout, a single polyvitamin tablet<sup>7</sup> was given to each member of the squad once a day, and for a mineral supplement two no. 0 capsules containing tricalcium phosphate and ferric phosphate were taken at each meal. These 6 capsules supplied 0.6 gm calcium, 0.3 gm phosphorus and 12 mg iron daily, in addition to the amounts contained in the food.

As the principal source of energy of the egg diet, a cornstarch muffin was baked of such a size that each provided approximately 207 Cal. The composition of this muffin—an evolutionary improvement on a previously used cornstarch "bread" (Sumner and Murlin, '38)—in an amount making 4 muffins, an average daily serving for one man, was as fol-

<sup>6</sup>For example, in the 1938 study (Murlin, Nasset and Marsh) referred to, there were 6 periods comprising a total of 30 days on the standard egg diet when the squad of 10 men showed an average daily excretion of only 0.77 gm fecal nitrogen. The diet contained an average total of 5.97 gm N. In a recent study (Hawley, Murlin, Nasset and Szymanski, '48) the average daily fecal excretion by 10 men on a protein diet for 7 days was 0.72 gm N. The diet contained only 0.290 gm N, aside from caffeine N amounting to 0.117 gm.

<sup>7</sup>Upjohn's "Unicap."

lows in grams: Cornstarch 100, crisco 27, baking powder 5, "4X" cream 33, salt 2, egg 33, sugar 10, water 46, and gum arabic 20. It was baked in muffin tins to a light brown color and proved superior in flavor as well as in texture to the formerly used "bread" or "biscuit."

The "4X" cream was obtained through priority order by the Office of Price Administration in Washington from a reliable local source. The eggs were delivered fresh each day

TABLE 1  
*The diet squad*

SUBJECT	AGE	HT.	SUR- FACE AREA	WEIGHT		CALORIES			
				Change		Average			
				Begin.	to end	Total	% distribution		
							P	CHO	F
	<i>yrs.</i>	<i>cm</i>	<i>m<sup>2</sup></i>	<i>kg</i>	<i>kg</i>		%	%	%
1 J.T.	25	169.7	1.75	65.9	— 0.5	3015	5	50.1	44.9
2 N.M.	29	178.0	1.91	74.4	+ 0.6	3338	5	48.9	46.1
3 R.B.	31	181	1.93	73.3	— 2.5	2907	5.4	51.6	43.0
4 J.S.	25	166.0	1.90	82.9	— 1.1	2892	5.1	50.4	44.5
5 N.T.M.	17	179	2.03	88.5	— 4.2	3637	5.0	49.7	45.3
6 R.G.	29	167.5	1.70	62.4	+ 0.6	2605	5.1	49.8	45.1
7 H.S.	21	179	1.90	72.4	— 0.8	3185	5.1	49.7	45.2
8 S.H.	18	179	1.83	66.5	— 2.3	3635	4.5	51.7	43.8
9 R.E.	18	169	1.71	63.8	— 2.1	3335	4.6	49.7	45.7
10 P.B.	30	189	1.87	64.4	+ 1.3	3064	5.0	50.0	45.0
11 L.E.	17	177	1.80	65.8	— 0.5	3435	4.7	50.0	45.3
Av.	24.7	175.6	1.85	70.9	— 1.0	3185	5.0	50.1	44.9

from a farm which regularly supplies the Strong Memorial Hospital and all other items of the diet were of standard high quality, though (because of market limitations during the war emergency) not always from the same source.<sup>8</sup> Because the experiment was conducted through the months of August and September, few of the squad preferred hot drinks with all their meals. A few took coffee regularly for breakfast, and tea for lunch or supper. A popular carbonated beverage<sup>9</sup> was

<sup>8</sup> Oranges were always of the "Sunkist" brand, lettuce the "Iceberg" variety, tomatoes of the "Beefheart" variety when possible, and so forth. The orange marmalade was supplied by the W. N. Clark Company, Rochester, N. Y.

<sup>9</sup> Coca-Cola.



permitted as a cooling drink as desired, in amounts up to two or three bottles daily. The nitrogen of the tea, coffee and carbonated beverage was regarded as completely excreted by the kidneys each day.

Aliquot samples of all foods were taken daily, and at the end of each 6-day period they were analyzed for nitrogen.

### *The diet squad*

For a result based on figures obtained with 10 persons it has been found advisable in diet work of this character to start with at least 11 experimental subjects. Some one person is likely to make a serious error in collection or to lose appetite because of the monotonous diet in one or more periods and, as occasionally happens in hot weather even with the utmost precautions, a mild food infection may occur, producing diarrhea. This happened in period IV of the present experiment, causing loss of stools by two subjects. In this instance it was possible to substitute the mean fecal N of the remaining 9 men without throwing the final results askew.

The squad, all males, was composed of three instructors, one graduate assistant, three medical students not qualified for the Army or Navy program, and 4 college freshmen enrolled as pre-medical students. Their physical characteristics and the average distribution of calories for each are given in table 1. All of these men were intellectually interested in the results and were in need of financial reward for close adherence to the dietary regime and quantitative collection of excreta. Before the end, one of the medical students was obliged to withdraw because of persistent alimentary distress occasioned by the large proportion of carbohydrate contained in the diet, and one of the freshmen for a valid reason was excused from the last period on the egg diet. The total squad was divided into two groups, a and b, which ate the several test proteins in reverse order (table 2). The purpose of this arrangement was to counteract any advantage which might accrue to one cereal food as against another by the accident of position in the series. For example, it requires at least 10 days on a low

TABLE 2

Order of the periods and number of subjects in each group (in parentheses)  
(All figures are reduced to daily values)

GROUP A					GROUP B				
Mean values for:					Mean values for:				
Urine N	Fecal N	Total excr. N	Diet N	N bal.	Urine N	Fecal N	Total excr. N	Diet N	N bal.
gm	gm	gm	gm	gm	gm	gm	gm	gm	gm
5.10	1.27	I Egg (6)	6.24	-0.13	4.98	1.51	I Egg (5)	6.25	-0.24
5.18	1.50	II Oats 1 (6)	5.72	-0.96	5.85	1.83	II Puffed mixed cereal (5)	6.41	-1.27
(6) 4.86	1.16	III Egg	6.65	+0.63	4.52	1.28	III Egg (5)	6.50	+0.70
(5) 4.68	1.17	IV Flaked wheat (5)	6.73	+0.87	5.13	1.56	IV Oats (5)	6.35	-0.34
(5) 5.90	1.96	V Egg	6.43	+0.56	4.07	1.33	V Egg (5)	6.37	+0.97
(6) 4.77	1.10	VI Oats 2 (6)	6.35	+0.39	5.68	1.91	VI Flaked wheat (5)	6.42	-1.17
(6) 4.81	1.15	VII Egg	6.47	+1.01	3.99	1.43	VII Egg	6.45	+1.03 (5)
5.57	1.47	VIII Puffed mixed cereal (5)	6.24	+0.87	4.33	1.40	VIII Oats 1 (4) <sup>2</sup>	6.91	+1.18 (4)
(6) 4.42	1.04	IX Egg (5)	6.15	-0.78	5.82	1.55	IX Egg (4)	6.70	-0.67
(5) 4.38 <sup>1</sup>	0.99		6.15	+0.73	4.12	1.63		6.80	+1.05
5.12	1.81								
4.39 <sup>1</sup>	1.03								

<sup>1</sup> Subject no. 5 ate egg in these two periods with group A but his egg periods belong to group B, period VIII.

<sup>2</sup> Subject no. 5 replaces no. 10 in this period and subject no. 9 is dropped because he had no egg period IX.

protein diet to reach a steady state in the rate of urinary excretion of nitrogen, because of the "deposit nitrogen" resulting from the previously relatively high protein diet. Therefore, as can be observed in table 2, which exhibits the average urinary N of all members in the successive egg periods, the average is 0.7 gm to 1.0 gm higher for the same number of subjects<sup>10</sup> in period I than in period VII, notwithstanding a somewhat higher ingestion of egg N in the later periods. This results in a higher N excretion in the first egg period than in any subsequent egg period, and in calculating the biological value the excess urinary nitrogen (cereal urinary N minus egg urinary N; see table 3) is less, as a percentage of the absorbed, in the first cereal period; consequently the biological value is higher than it should be. When, however, half of the squad eats the same cereal protein in the last test-protein period, the excess nitrogen is higher than it would be for them in the first cereal period, and consequently the biological value is lower. The advantage of the first position is offset by the disadvantage of the last. The gradual decline in average urinary N in successive egg periods is due to the fact that all cereal proteins have a lower B.V. than whole egg protein, and because of the accumulating deficit the egg protein is retained better as the series progresses.

## RESULTS

### *a. Biological value*

This measure of the nutritive value of a protein is defined briefly as the percentage of the absorbed N which is retained in the body. It differs from the egg replacement value in two ways: (a) It takes account of the relative alimentary losses on the standard protein and test protein, and, assuming that the former loss is minimal, it thus measures absorbed N by deducting the fecal food N for the test protein (obtained

<sup>10</sup> It will be observed that the average data for several egg periods had to be calculated for two different numbers of subjects, depending on the number who completed the adjacent cereal period successfully.

by the difference between fecal losses on the two proteins) from the fed N. In this study the assumption was thwarted in the case of one subject, who did not digest egg protein perfectly in *any* period, as was evident from obvious traces of it in the stools; (b) it takes account of metabolic excess N by deducting the urinary excretion of nitrogen on the egg protein diet from that on the cereal protein diet. All other nitrogenous constituents of the two diets being the same, this excess is a measure of the failure of the test protein to equal the egg protein in its value (supplemented by the other sources of nitrogen) to the tissues, as gauged by retention.

Theoretically, one should obtain biological values, and replacement values as well, by comparison of an intermediate test protein period with the preceding and following egg proteins. In this study, however, as in a preceding one on breads (Murlin et al., '41), it was noted that the average retention in each succeeding egg protein period was greater than in the one before. For this reason it was decided to employ the mean fecal and urinary nitrogen values from all the egg periods as the basis of calculation of biological values, instead of the data from the two periods adjacent to each test protein period. This was not done, however, before comparing one method with the other from the standpoint of concordance, or, in other words, determining which method resulted in the least variation among the several subjects as shown by the standard deviation. In fact, one other method was compared with these two by the same criterion, this method involving the use of the egg period immediately following the cereal period, thus eliminating the first egg period which, as noted above, gave clear evidence of a "hangover" effect from the previous higher protein diet. Neither of the two latter methods proved so satisfactory as the first, for their standard deviations were greater. The downward trend in the excretion of nitrogen from the first to the last egg period thus is compensated for not only by feeding the cereals in reverse order to the two groups of the squad (table 2), but also by averaging all the egg periods using a common plane

TABLE 3  
*Mean relative biological values for cereal proteins*

DATUM OF INTEREST	CEREAL			
	Oats no. 1	Oats no. 2	Puffed Mixed cereal	Flaked wheat
No. of subjects	10	10	8	10
a. Cereal fecal N (gm)	1.56	1.52	1.89	1.94
b. Egg fecal N (gm)	1.20	1.27	1.27	1.28
c. (a-b) Difference (gm)	0.36	0.25	0.62	0.66
d. Total food N (gm)	6.07	6.38	6.34	6.44
e. (d-c) Absorbed N (gm)	5.71	6.13	5.72	5.78
f. True digestibility (%)	93.9 $\pm$ 3.3	96.1 $\pm$ 4.9	90.2 $\pm$ 3.5	89.7 $\pm$ 2.6
g. Cereal urine N (gm)	5.21	5.28	5.57	5.78
h. Egg urine N (gm)	4.63	4.54	4.58	4.44
i. (g-h) Excess N (gm)	0.58	0.73	0.99	1.35
j. (e-i) Retained N (gm)	5.13	5.40	4.73	4.43
k. $\frac{j \times 100}{e} = \text{Biol. val. (\%)} $	89.9	88.1	82.8	76.7
l. Mean deviation from mean biol. val. (gm)	$\pm$ 3.0	$\pm$ 3.9	$\pm$ 4.4	$\pm$ 4.5
m. Standard deviation (gm)	3.81	5.56	6.52	5.85

Significance of differences between mean biological values:

- (1) No. 1 Oats and puffed mixed cereal.  $n = 18$ , " $t$ " = 6.0,  $P = .015$ .
- (2) No. 1 Oats and flaked wheat.  $n = 18$ , " $t$ " = 6.0,  $P = .0001$ .
- (3) No. 2 Oats and flaked wheat.  $n = 18$ , " $t$ " = 4.47,  $P = .0002$ .
- (4) Flaked wheat and puffed mixed cereal.  $n = 16$ , " $t$ " = 2.03,  $P = .06$ .

of reference. Each cereal food, therefore, occupies the same average distance from the midpoint sequentially, and is topographically oriented to the same fundamental datum.

In calculating biological values and the statistical significances between mean values for the different cereals it was necessary to exclude extreme variants in all the data for the 4 cereals — one each for the two oats cereals and flaked wheat, and two for the puffed mixed cereal. Only those which fulfilled the Chauvenet criterion for rejection of extreme variants in small populations (as this is set forth by Davenport and Ekas, '36) were discarded.

The significances of the differences between pairs of means, according to Fisher's method, are shown in the footnote to table 3. It is evident that the two whole oat products give higher biological values than the more extensively pretreated

ones, and that in all comparisons but one the differences between means are highly significant.

The true digestibilities of the several cereals compared with those of all the egg periods averaged together do not, however, exhibit differences of any significance. The differences become very significant when they relate to what happens after absorption. Observe by how much more the cereal nitrogens exceed the egg urine nitrogens in the last two columns of table 3 than in the first two. This table also shows how the differences are subtracted from the absorbed nitrogens to get the nitrogens retained (line j) and how the latter are much less for the last two columns, thereby giving lower biological values (line k).

The cereal fecal nitrogens in the last two columns exceed the basal egg fecal nitrogens by nearly twice the same difference in the first two (line c). This results in lowering the amount of absorbed nitrogen, and explains the lower digestibilities of the two cereal products represented. By the same token, the lower absorption should serve (other factors being equal) to raise the biological values, but the greater excess of urine nitrogen from the same two cereals means less retention, and that diminution more than offsets the effect on absorption.

#### *b. Egg replacement values (E.R.V.)*

As the term is used in this laboratory, the E.R.V. is calculated from the crude N balance data, as shown in table 4. It may be defined as the extent, percentage-wise, to which any particular protein can replace egg protein *in maintaining N balance*. Ideally, the egg and test protein should be fed at exactly the same levels and, as may be seen in table 4, this ideal was approximated very closely in tests with all but the first cereal listed. This particular failure was due to using a wrong value for the nitrogen in this cereal. An adjustment in the difference between N balances was therefore made, upon the reasoning that if a lower intake of cereal protein produced a given minus N balance, a higher intake

(equal to that of the egg period) would have produced a lesser minus N balance in inverse proportion to these levels of intake. Thus, in the present instance, for group a,  $6.44:5.72 :: 1.22 : x$ , and  $x = 1.08$ . This corrected difference in N balances weighted against a similarly adjusted difference in balances for group b gives the weighted final difference between balances, which determines the extent to which the cereal failed to replace egg protein.<sup>11</sup> This difference of balances, expressed as a percentage of the egg N ingested and subtracted from 100, is the extent to which the cereal protein *did* replace egg protein. A similar small adjustment was made for the results with a and b groups on all cereals where significant differences in N intake existed.

It is evident from table 4 that the two whole oat products excel in their ability to replace egg protein and in the maintenance of N balance, and that the two more extensively pretreated products—one from wheat and the other from a mixture of the three cereals, oats, corn and rye fall considerably below this level. In order to say exactly how much the wheat product was reduced in this capacity, a whole wheat in its natural form or with a minimum amount of pre-treatment should have been tested in parallel experiments with the same squad, as was done in our earlier study (Murlin et al., '38) and by Kuether and Myers ('48). However, it is of interest that we obtained a replacement value with this product only a few points above that found in 1938, which may be explained by the fact that the ingestion level of nitrogen averaged 0.6 gm higher than in the earlier study. No such combination of oats, cornmeal and rye in natural form was available for comparison with the puffed mixed cereal.

<sup>11</sup> Such an adjustment leaves something to be desired, of course, because failure of a given protein to maintain N-balance is due presumably to the critical level at which one or more of the essential amino acids fails to suffice for some governing protein synthesis or syntheses. We are not yet in possession of sufficient information regarding these critical levels to make adjustments on the basis of known amino acid compositions. Studies like those of Block and Mitchell ('46) show the direction of advance in these matters.

TABLE 4  
Egg replacement values of cereal proteins

GROUPS	CEREAL FOOD	FOOD N				N BALANCES				NO. OF SUBJ.	WGTD. FINAL AV. DIF.	% OF MEAN EGG N	% REPLACEMENT OF EGG N	ST. DEV.
		1	2	3	4	5	6	7	8	9	10	11	12	13
					Weighted mean of egg periods	Before and after periods	Difference	Cereal	Mean of egg periods before and after	Difference	ADJUSTED DIF.			
a	Oats no. 1	5.72			6.60	{ 6.44	-0.72	-0.96	+0.26	1.22	1.08	6	1.34	79.6
b		6.70				{ 6.85	-0.15	-0.67	+1.11	1.78	1.74	4	20.4	7.2
a	Oats no. 2	6.42			6.42	{ 6.41	+0.01	-0.62	+0.69	1.31	1.31	6	1.24	8.3
b		6.35				{ 6.43	-0.08	-0.33	+0.83	1.16	1.15	5	19.3	80.7
a	Puffed mixed	6.15			6.28	{ 6.19	-0.04	-0.78	+0.80	1.58	1.57	5 <sup>1</sup>	1.54	5.9
b	cereal	6.41				{ 6.37	+0.04	-1.27	+0.23	1.50	1.51	5	24.5	75.5
a	Flaked	6.45			6.49	{ 6.58	-0.13	-1.41	+0.71	2.12	2.08	5 <sup>2</sup>	2.12	67.3
b	wheat	6.42				{ 6.41	+0.01	-1.17	+1.00	2.17	2.17	5	32.7	6.6

<sup>1</sup> Number 5 is excluded from this group as an extreme variant.

<sup>2</sup> Number 4 is excluded from this group as an extreme variant.



The greatest differences between means in the replacement values shown in column 13 of table 4 are those between the two whole oats products and the flaked wheat. The difference between the means of no. 2 oats and the puffed mixed cereal is just short of statistical significance for 11 and 10 subjects, respectively.

#### DISCUSSION

The results of our experiments confirm the findings of Kuether and Myers ('48) for their several experiments on two products, which presumably are in all particulars the same as those used in this study. Rolled oats, in the experience of this laboratory, gives the same replacement value whatever its trade name. It is, however, possible that the product the above workers call "exploded oats" has undergone some change in formula or manufacturing process from the one called "puffed mixed cereal" used in this laboratory 5 years earlier.

It is puzzling to the present authors why, in collection periods of 10 to 12 days with cereal as the sole source of nitrogen in the diet, Kuether and Myers did not obtain any closer agreement in nitrogen balances than has been obtained in this laboratory in periods of 6 days each on three cereals of identical nature supplemented by milk and fruit nitrogen. The impression is given in their table 6 of their having struck a N balance every single day for each subject, whereas from the description of their method it is clear that stools were separated only in periods of several days and that a single value for the period was obtained by dividing by the number of days. The variable in known excretion values from day to day, therefore, can only be the urine nitrogens. It is decidedly misleading to give daily variations in *balance* as "standard deviations" when they are really variations only in the urine nitrogen. The number of subjects referred to in their table 6 is only three, and there are obvious deviations among them that in sum amount at times to more than the mean value shown, so that the mean deviation from the mean is more than 50%

of the mean. This is true of the "exploded oat cereal" and the whole wheat, which gave the lowest mean nitrogen balances for the three subjects. The "standard deviations" given for each subject to indicate day-to-day variations, also are at times higher than the mean of "nitrogen balances." It would have been of interest, but of no more general application, if the authors had merely compared one subject with another.

The opinion expressed in a former paper (Murlin et al., '38) that wheat flakes exhibited a reduction in biological value due to heat treatment appears to be confirmed by the present results, for the value reported in table 3 is considerably lower (76.7 *cf.* 82.1) than that reported earlier, although the heat treatment appears to be less severe (see p. 406).

There is confirmation also of the reduced biological value for the growth of rats of the puffed mixed cereal reported by Stewart et al. ('43) in the low B.V. of this protein for maintenance in man as shown in table 3. There are no results in this communication confirming an earlier finding that undamaged oats are superior to undamaged wheat in biological value or replacement value. Kuether and Myers ('48), however, seem to stand alone in their findings of the reverse.

#### SUMMARY

Four cereal products prepared as "breakfast foods" have been studied with reference to the biological value for human subjects of the contained proteins based on egg protein as standard. Two of the products were whole oat preparations; one, three-quarters oats; and one, whole wheat with "all of the original nutrients retained."

The egg replacement method as previously employed in this laboratory was followed and some improvements were made. The squad was divided into two groups, which ate the cereals in reverse order, thereby counteracting any advantage from mere position in the series of 9 periods — 5 on egg, 4 on cereals.

The biological values of the cereal proteins were calculated for each individual on the basis of his average data from all

the egg periods. Hence, each cereal food occupied the same average distance from the nitrogenous midpoint of the series of standard diets.

After calculation of the biological values and the replacement values in terms of the egg standard, and obtaining standard deviations, the extreme variants for each cereal were eliminated in accordance with standard statistical procedure.

The biological values relative to egg protein of the 4 cereal products recalculated with their standard deviations for the number of subjects retained were found to be as follows: no. 1 oats 89.9 (3.81), no. 2 oats 88.1 (5.56), puffed (or "exploded") oats-corn-rye cereal 82.8 (6.52), and flaked wheat 76.7 (5.85). The differences between the means for the two oats products and wheat flakes are highly significant, as is also true for the no. 1 oats and the puffed oats-corn-rye cereal but not for the latter and wheat flakes.

The replacement value, defined as the extent to which any particular protein can replace egg protein in the maintenance of nitrogen balance, was calculated for each group of subjects for each cereal. The order of values derived from weighted differences in average N balances between cereal and egg are as follows: no. 1 oats 79.6 (7.2); no. 2 oats 80.7 (8.3); puffed oats-corn-rye mixture 75.5 (5.9), and wheat flakes 67.3 (6.6). Only the differences of means between the two whole oats products and wheat flakes are statistically significant.

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# IMPROVING THE NUTRITIVE VALUE OF FLOUR

## III. THE USE OF ENRICHED AND NON-ENRICHED FLOUR IN DIETS SIMILAR TO THOSE CONSUMED BY CERTAIN LOW-INCOME GROUPS IN SOUTH CAROLINA <sup>1</sup>

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### FOUR FIGURES

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Experiments have been reported (Westerman and Bayfield, '45; Westerman and Hall, '47; Guerrant and Fardig, '47; Westerman and Templeton, '48) in which enriched and non-enriched flour were used as the sole source of the B vitamins in the diets of experimental animals. In these experiments a fairly purified diet was used. The human dietary contains other foods besides cereals which furnish B vitamins; it seemed likely, therefore, that using diets made up of natural foods comparable to those consumed by human beings would give added information relating to this problem. The diets used in the experiments described in this report were patterned after those consumed by people with poor food habits or with low incomes. Such groups of individuals are most likely to include considerable quantities of cereals in their

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diets, either from habit or from economic necessity, since such foods represent cheaper sources of energy.

#### EXPERIMENTAL PROCEDURE

Albino rats weighing between 45 and 55 gm, and 28 days of age, were depleted of their body stores of B vitamins by placing them on a B-complex-free diet for 10 to 14 days. The animals were grouped in such a way as to have an equal distribution in regard to sex and litter mates. Six or more animals were placed on each diet.

The diets were patterned after those described by Moser ('45) as being consumed by some of the rural families in Pickens County, South Carolina. These data were calculated on the basis of daily consumption per individual, and the diets for the rats were based on the amount of each of the different foods consumed per person per day. In order to facilitate the preparation of the food, it was necessary to make some modifications in the diets and to group certain foods together. Carrots were used to represent yellow vegetables, and green beans, spinach, cabbage and broccoli were used alternately as the green vegetables. An average of the nutritive values of these vegetables was used in the calculations. In order to have a fair representation of the meat used in the average diet, beef and pork were mixed equally. Apples were used as representative of the fruits in the diet and dried beans and peas were the legumes. Dried whole milk was used in order to reduce the liquid content of the diet. The fats were margarine and salad oil. All cereals were added to the diets as enriched flour, non-enriched flour, ground whole wheat or cornstarch. The whole wheat was included in order to make a comparison of the diets having a whole grain cereal as against those containing enriched or non-enriched flour. A purified carbohydrate, cornstarch, replaced the cereal in some of the diets, in an attempt to discover by such replacement whether other constituents of the diets were furnishing enough B vitamins for adequate nutrition. The foods requiring cooking were cooked. The wet ingredients were mixed

and finely divided in a blender and then mixed with the dry ingredients in such a way that the diets were homogeneous. They were prepared once each week and kept in frozen storage until needed.

Calculations of the nutritive values of the different diets were made by using an average of the values by Sherman ('46) and Turner ('46), except for the flour and wheat; these were assayed. The enriched flour contained 6.94, 3.00 and 1.15  $\mu\text{g}$  per gram of thiamine, riboflavin and pantothenic acid, respectively; the non-enriched flour 1.35, 0.74 and 1.12  $\mu\text{g}$  per gram; and the whole wheat 5.15, 1.06 and 3.19  $\mu\text{g}$  per gram of these same vitamins.

Four different experiments were conducted using cereals to furnish 62, 50, 40 and 33% of the total calories in the diets. It might seem that 62% of the calories from cereals is a rather large amount and it may be that people in the United States do not as a rule consume cereals in this amount, but there are countries in Europe where cereals are the main source of calories. Moser ('45) has shown that some of the people in South Carolina do consume cereals at levels of 40 and 50% of the total calories, and cereals may furnish 30% of the calories in other American diets. The constituents of the diets and the percentage of calories supplied by each are shown in table 1. The growth studies were carried out over periods of 9 to 14 weeks, and in some cases through the second generation.

#### RESULTS AND DISCUSSION

##### *First experiment: Cereals furnished 62% of the total calories*

In experiment I the cereals in the diets were as follows: diet I contained enriched flour, diet II non-enriched flour, diet III ground whole wheat and diet IV cornstarch. Growth studies were conducted over a period of 9 weeks and figure 1 gives the average weekly weight gains made by the animals on the different diets. The rats on diet I with enriched flour furnishing 62% of the total calories in the diet gained on the

average 17 gm more than those on diet II with non-enriched flour. Those on diet III with whole wheat gained 6 gm more than those on the diet with the enriched flour. The animals on diet IV containing cornstarch made the least gains during the 9-week period.

A comparison of the nutritive values of the 4 diets is shown in table 2. The nutritive values of all the diets except the one containing cornstarch were well within the range of those established as adequate for the normal person. The

TABLE 1

*Composition of diets based on the daily food consumption of humans*

FOOD	EXPERIMENT I		EXPERIMENT II		EXPERIMENT III		EXPERIMENT IV	
	Calories	Cal.	Calories	Cal.	Calories	Cal.	Calories	Cal.
		%		%		%		%
Cereal	2396	62.0	1200	40.0	888	32.8	1122	49.9
Sugar	143	3.7	661	22.0	398	14.7	131	5.8
Fat	432	11.2	220	7.2	432	15.9	388	17.2
Meat	142	3.7	114	3.8	142	5.2	142	6.3
Milk	372	9.6	298	9.9	372	13.7	166	7.4
Eggs	24	0.6	79	2.6	24	0.9	21	0.9
Legumes	158	4.1	140	4.7	98	3.6	42	1.9
Potatoes	58	1.3	150	5.0	213	7.8	131	5.8
Carrots	45	1.2	40	1.2	45	1.7	22	1.0
Apples	64	1.7	63	2.1	64	2.4	61	2.7
Green vegetables	33	0.9	31	1.0	33	1.2	25	1.1

diets containing enriched flour and whole wheat were considerably higher in the B vitamins and iron than the other two diets. This probably accounts for the greater average weight gains of the rats on these diets. The low protein content of diet IV containing cornstarch, as well as the smaller quantities of the B vitamins, undoubtedly were responsible for the slow growth of these animals. The protein deficiency was corrected in the experiments to follow by including vitamin-free casein in the diet along with the cornstarch. The results of this experiment would seem to indicate that enriched flour is superior to non-enriched flour in supporting



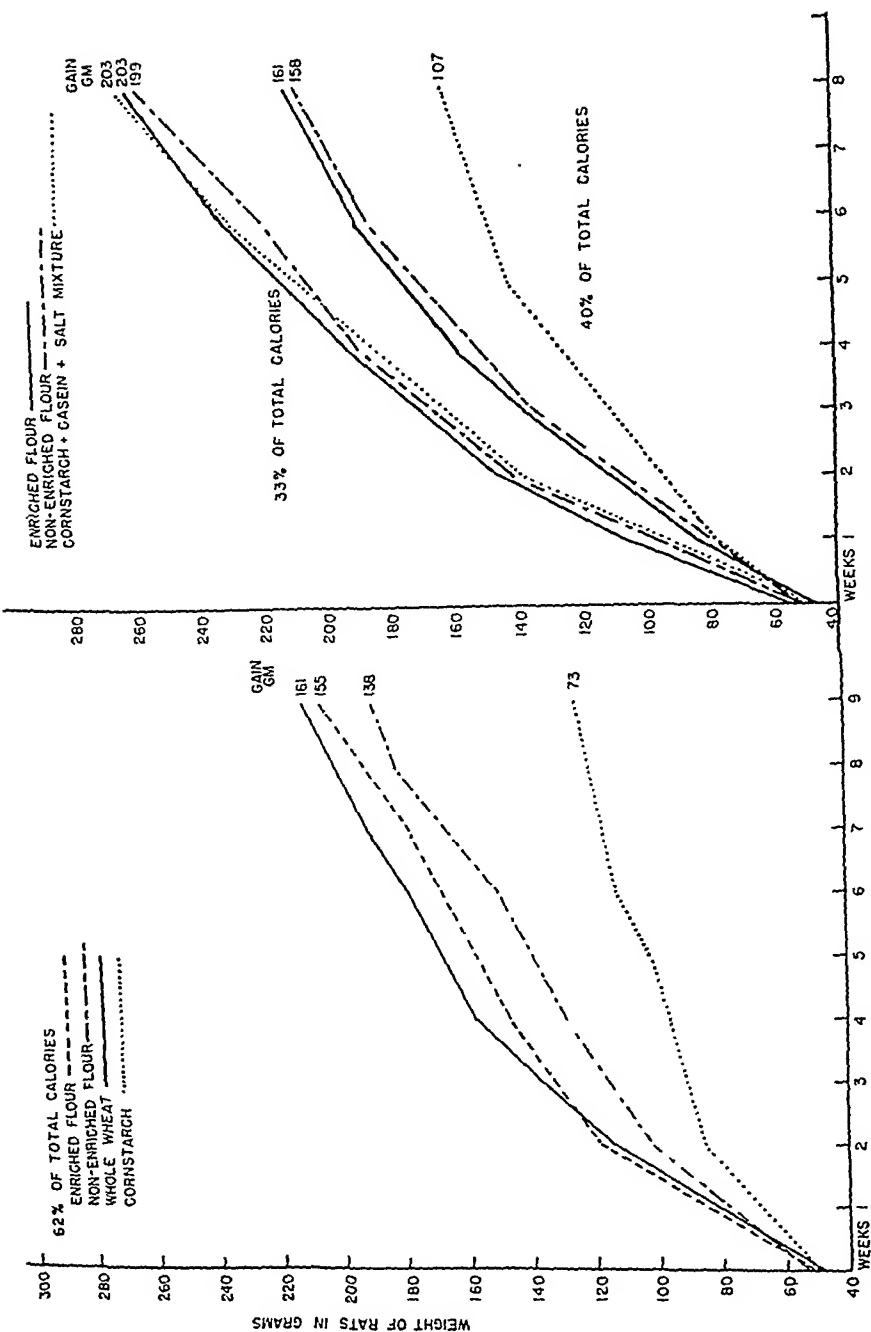


TABLE 2  
*Nutritive value of daily diets as consumed by humans*

DIET		Calo- ries	Prot.	Ca	Fe	Thia- mine	Ribo- flavin	Nicotinic acid
			<i>gm</i>	<i>gm</i>	<i>mg</i>	<i>mg</i>	<i>mg</i>	<i>mg</i>
Experiment I: 62% of total calories from cereals								
I	Enriched flour	3867	118	1.11	29.6	5.75	3.69	29.3
II	Non-enriched flour	3867	118	1.11	14.7	2.04	2.13	11.1
III	Whole wheat	3908	127	1.26	36.3	4.74	2.47	43.5
IV	Cornstarch	4171	45	0.93	10.0	1.11	1.67	5.7
Experiment II: 40% of total calories from cereals								
I	Enriched flour	2996	81	0.86	20.0	2.61	2.48	18.6
II	Non-enriched flour	2996	81	0.86	13.0	1.36	1.71	9.5
III	Cornstarch + vitamin-free casein	3148	85	0.80	11.0	1.09	1.57	6.8
Experiment III: 33% of total calories from cereals								
I	Enriched flour	2709	67	0.97	17.0	2.32	2.36	16.6
II	Non-enriched flour	2709	67	0.97	11.0	1.29	1.78	9.8
III	Cornstarch + vitamin-free casein	2722	67	0.93	10.0	1.19	1.68	7.8
Experiment IV: 50% of total calories from cereals								
I	Whole wheat	2270	66	0.62	18.1	2.51	1.28	23.4
II	Enriched flour	2251	62	0.52	15.1	2.98	1.85	16.8
III	Enriched flour + salt mixture	2251	62	1.15	31.4	2.98	1.85	16.8
IV	Enriched flour + casein	2371	92	0.52	15.1	2.98	1.85	16.8
V	Enriched flour + casein + salt mixture	2371	92	1.15	31.4	2.98	1.85	16.8
VI	Cornstarch + casein + salt mixture	2513	58	1.10	22.2	0.80	0.90	5.7
VII	Non-enriched flour	2251	62	0.52	8.1	1.24	1.12	8.2
VIII	Non-enriched flour + salt mixture	2251	62	1.15	24.4	1.24	1.12	8.2
IX	Non-enriched flour + casein	2371	92	0.52	8.1	1.24	1.12	8.2
X	Non-enriched flour + casein + salt mixture	2371	92	1.15	24.4	1.24	1.12	8.2

growth in rats when these materials furnish 62% of the total calories of the diet.

*Second and third experiments: Cereals furnished 40 and 33% of the total calories*

In these experiments it was decided to lower the amount of cereal in the diets and to test the effect of the inclusion of more milk, fruit, and vegetables. The calories obtained from sugar were increased in experiment II and those from fat in experiment III. The composition of the diets is shown in table 1 and the nutritive value in table 2. Three diets were used in each experiment: diet I, containing enriched flour; diet II, containing non-enriched flour; and diet III, containing cornstarch with vitamin-free casein added to bring the protein level of this diet up to that of the other diets.

The results of the 8-week growth test are shown in figure 2. It may be noted that the animals on the diets with 40% of the calories from enriched and non-enriched flour maintained the same growth rate, but those with cornstarch at the same level gained on the average much less. The nutritive values of the diets (table 2) show that diet I, containing enriched flour, had a higher content of iron and the B vitamins, but it appears that diet II, with non-enriched flour, supplied enough of these materials to support the same amount of growth while diet III, containing cornstarch, did not.

In experiment III with 33% of the total calories from enriched and non-enriched flour, and from cornstarch with vitamin-free casein added, the growth rate of the rats (fig. 2) on all three diets was approximately the same. There were some differences in the nutritive values of these diets as shown in table 2, but evidently the materials in the diets furnished a better source of all the nutrients than did those in experiment II. The rats on these diets also made much better gains than did those with 40% of the calories coming from enriched or non-enriched flour. These animals with 33% of the calories from cereals also obtained a larger per cent of

calories from fat, meat, milk, and potatoes than those at the 40% level, and this may account for the better growth, as fat would have a sparing action on carbohydrate metabolism and meat and milk would furnish more of the B vitamins. These results indicate that when meat, milk, and vegetables are present in adequate quantities in the diet and there is less consumption of cereals, the benefits from the use of enriched flour are not easily demonstrated. This was also shown by Winters ('47), who fed rats on diets representative of the average American diet with 30, 33, and 36% of the total calories derived from cereals. No significant differences in the growth of the animals fed the enriched and non-enriched cereals at these levels were noted.

*Fourth experiment: Cereals furnished 50%  
of the total calories*

The data on food consumption of low-income groups as given by Moser ('45) did not allow for any waste of food. It was expected, however, that there would be some waste in preparing the fruits and vegetables and that all the food placed upon the table would not be eaten. It was decided that at least 10% of the estimated amount of food taken into the homes would be wasted as far as human consumption was concerned. Therefore the food consumption was recalculated for the purpose of the present study on the basis of a 10% loss. The milk was reduced from 60 to 30 gm. While a rural population may have more milk in the diet, it was thought urban families on low incomes would have about one-half as much.

With the above modifications, the cereal component of the diets furnished about 50% of the total calories. In this experiment some diets with vitamin-free casein and salt mixture added were included, so that protein and minerals would not be deficient. The vitamin-free casein provided 5.1% of the total calories and the salt mixture made up 0.5% by weight of the total diet. The constituents of the diets and the percentage of calories supplied by each are given in table 1, and the nutritive values in table 2. It may be noted that the

protein content of the diets with added casein is about one-third greater than that of the other diets. The calcium content of the diets without the added salt mixture was inclined to be low, due in part to the small amount of milk, which approximated one cup per day per individual. The iron content of diets VII and IX was low due to the use of non-enriched flour. Diets VI through X, containing cornstarch and non-enriched flour, were much lower in the B vitamins than diets I through V which contained the enriched flour.

The average weekly weight gains of the animals on the different diets are shown in figure 3. For purposes of comparison the weights of the animals with enriched flour in the diet were grouped with those on the corresponding diet containing non-enriched flour. As is shown by the growth curves in figure 3, the diets containing the enriched flour and the whole wheat supported about the same rate of growth in the rats, as both these groups made an average weight gain of 239 gm during the 13-week period. Those animals with non-enriched flour in the diet grew at a slower rate and gained a total of 229 gm during the same period. Those with cornstarch replacing cereal in the diet showed the least gain, 186 gm, even though this diet contained vitamin-free casein and salt mixture. It would seem that the B vitamins contributed by the enriched flour played an important part in the growth of the rats, and were factors in promoting slightly better growth in animals having enriched flour in the diet as compared with those on the diet containing non-enriched flour.

When salt mixture was added to the diets containing enriched and non-enriched flour, the weight gains were 20 gm more than without the added salt mixture, but there was an 11-gm difference in the gains made when enriched flour was in the diet over those made on non-enriched flour. When vitamin-free casein was added to increase the protein content of the diet, the growth rate was increased on both diets but the rats on enriched flour made slightly better gains.

When both salt mixture and protein were added, the gains were even greater but there was some difference between the

weight gains of the animals on the enriched flour diet over those rats on the non-enriched flour. It is likely that if more milk were included the diet containing non-enriched flour would be more nearly adequate in minerals and proteins as well as the B vitamins.

The results of the growth test indicate that enriched flour aids in promoting growth when milk and vegetable consumption is lowered and cereal consumption increased. The addition of the mineral salts and protein to the diet was beneficial, but slightly better growth rates were obtained with the use of enriched flour in either of these diets. It seems likely that the rat, being a fast growing animal, requires large quantities of minerals and proteins.

At the end of 13 weeks on the test diets the males and females were allowed to breed. The females on the diet containing enriched flour gave birth to more young than did those with non-enriched flour in the diet. For some unexplained reason none of the young of the mothers on diets II and VII lived. The females on diet III containing enriched flour and salt mixture, and those on diet VI containing cornstarch, salt mixture and casein, raised 15 young; however, the young of rats on diet III had an average weight of 55 gm at 28 days of age while those of animals on diet VI weighed 38 gm. A comparison of the young of rats on diet V, containing enriched flour, salt mixture, and casein, with those of animals on a similar diet containing non-enriched flour shows approximately the same number of young raised, but those of females with enriched flour in the diet weighed on the average 12 gm more than those of rats on the other diet. These data indicate that enriching the flour aids in reproduction and in the raising of normal young.

When the young rats of the second generation were 28 days old they were divided into groups of 6 with equal distribution as to sex and litter mates, and kept on some of the same diets. Since the females on the diets containing enriched and non-enriched flour did not raise their young, it was necessary to transfer rats from the other groups to these diets. The

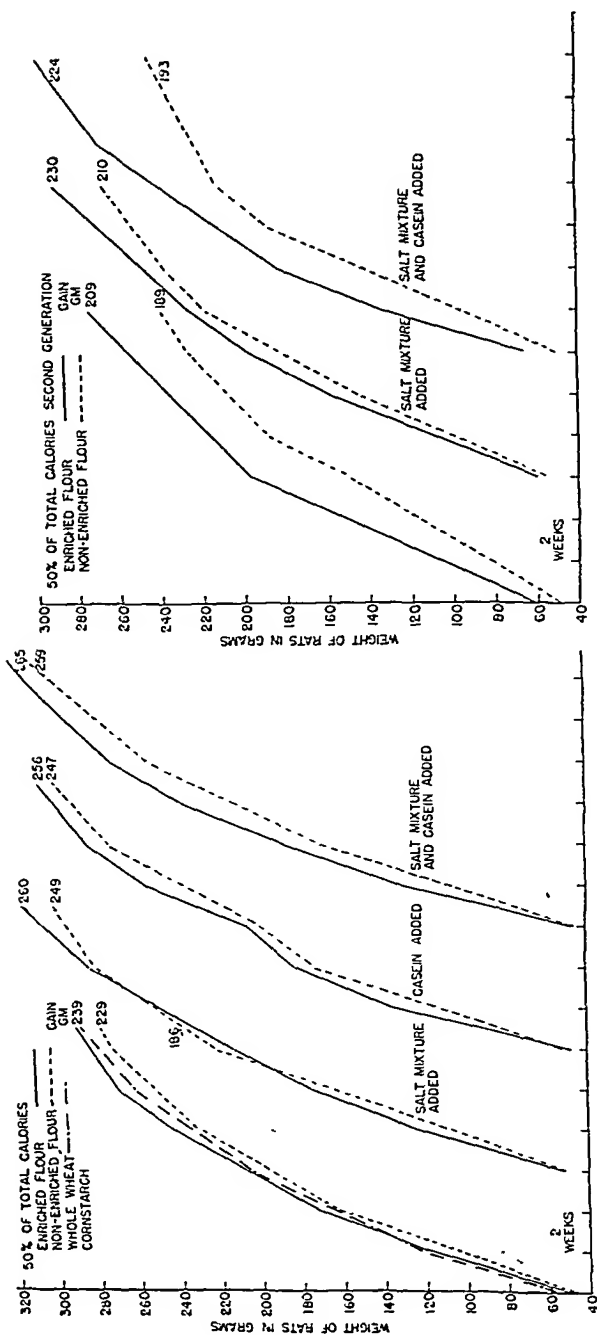


Fig. 3 Growth curves of first generation animals in experiment IV.

Fig. 4 Growth curves of second generation animals in experiment IV.

growth test was conducted over a period of 14 weeks so that, for purposes of comparison, the rats would be the same age at the end of the test as those of the first generation which had been placed on a depletion diet for one week before starting the test. Figure 4 shows the differences in the average total gains among the rats on the different diets. The rats on the diet containing enriched flour gained on the average 20 gm more than those on the diet with non-enriched flour; those on the diet with the enriched flour and salt mixture also gained 20 gm more than those with the non-enriched flour and salt mixture in the diet. The greatest difference in average weight gains was shown by the animals on the diets containing enriched flour with salt mixture and vitamin-free casein, and the non-enriched flour with salt mixture and vitamin-free casein added. Those animals receiving the enriched flour gained on the average 31 gm more than those on a similar diet with non-enriched flour. The addition of the protein did not improve the growth rate, since the rats with salt mixture included in the diets made better gains than those with both salt mixture and protein added. However, the test with the second generation animals showed that for purposes of growth enriched flour is superior to non-enriched flour as a source of the B vitamins when the flour is fed at a high level.

#### SUMMARY

Combinations of foods similar to those consumed by human beings were fed to albino rats to determine the significance of the use of enriched flour in the diets. The results indicate that when 62% of the total calories were furnished by flour, the animals with enriched flour in the diet gained on the average 17 gm more during a 9-week growth period than did those on diets containing non-enriched flour. When 50% of the total calories were furnished by flour, the animals with enriched flour in the diet gained an average of 10 gm more than those receiving non-enriched flour. Even when salt mixture was added to increase the calcium content of the diet, or vitamin-free casein was added to increase the protein, or when both



of these were added to the same diets, the rats receiving the enriched flour made the greater gains. The second generation animals showed a wider variation, as those fed enriched flour gained on the average 20 gm more than those with non-enriched flour in the diet.

The average weight gains made by the animals with 33 and 40% flour in the diet, respectively, showed no differences between those receiving enriched and non-enriched flour. The animals with 33% of the calories from enriched and non-enriched flour made better weight gains than those whose diets included flour at the 40% level. This was due probably to the added milk, meat, and vegetables in the former diet, which replaced the cereals.

The results indicate that when flour is consumed in large quantities the enriched product aids in promoting a better growth rate in rats than the non-enriched form.

#### ACKNOWLEDGMENT

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## RIBOFLAVIN METABOLISM OF YOUNG WOMEN ON SELF-SELECTED DIETS

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Relatively few riboflavin metabolism studies using young women as subjects have been made. So far as is known, none has been made solely with subjects on a self-chosen diet, although several have observed urinary excretions. The 24-hour urinary riboflavin excretion values for university women students and faculty on unrestricted diets reported by Strong et al. ('41) were of the order of 500 to 800  $\mu\text{g}$ . Brewer and co-workers ('46) observed the urinary excretion of riboflavin by 20 college women on self-selected diets over a three-day preliminary period. The range in urinary riboflavin was from 60 to 920  $\mu\text{g}$  in 24 hours. This wide range is to be expected from women consuming different diets, although the dietary riboflavin was not determined. Similarly, Davis, Oldham and Roberts ('46) observed the urinary excretion of riboflavin when young women ingested self-chosen diets during a preliminary period. They also obtained a wide range of values, with an average daily urinary excretion of 433  $\mu\text{g}$ .

Few studies showing the fecal excretion of riboflavin have been reported. Riboflavin is synthesized in the intestine, and therefore fecal excretion does not measure the unused portion of the ingested vitamin. The study of Davis and co-workers ('46) indicated that the fecal excretion of riboflavin is not

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markedly influenced by the dietary level unless the diet is an unusual one. Hathaway and Lobb ('46) found that a natural diet favored the synthesis of riboflavin more than did a synthetic diet, whereas Denko et al. ('46) reported that men on a diet restricted in protein and B vitamins showed a higher fecal output of riboflavin than when on a normal diet.

The purpose of this study was to determine the riboflavin value of food and excreta of young college women consuming a self-selected diet from a common food supply.

#### PROCEDURE

Nine young college women between the ages of 18 and 25 participated in this study. The precautions and procedures used in collecting the food and excreta were those previously reported by Holt and Scoular ('48). However, for this study the jars used for food and feces were painted on the outside with black paint to prevent light destruction of riboflavin. The amber bottles used for urine were found to give adequate protection without painting.

One Eastman Safelight lamp carrying a 20-watt bulb and equipped with a Wratten light filter was the only illumination used during the entire period of analysis.

Duplicate samples, 5 or 6 gm, of the macerated food were taken for analysis. The method employed for milk, food and feces was essentially that of Conner and Straub ('41), except that the adsorbent was washed free of dust and adsorbed gases with 2% acetic acid and repeated rinsings with distilled water, as recommended by Ferrebee ('40). The volume of the eluant necessary to free the riboflavin from the adsorbent was 80 ml for the samples used in this study, as compared to 50 used by Conner and Straub ('41). Filters B<sub>2</sub> and PC<sub>2</sub> with which the Coleman Photofluoremeter was equipped, were used for transmitting the fluorescent and incident light.

A complete blank consisting of all the reagents used throughout the method was also carried through the entire procedure. The value for the blank was subtracted from the fluorescence reading of each sample before determining the riboflavin value

from the calibration curves made with pure riboflavin. If the duplicate samples checked within a plus or minus 10%, the average was used in determining the total riboflavin content. Two curves were made, since two methods were used in determining the riboflavin values. Conner and Straub's method ('41) was used with modifications previously mentioned for the samples of milk, food and feces, while Ferrebee's method ('40) was used for the analysis of the duplicate urine samples. With the latter method it was also necessary to increase the eluant; in this case, to 60 ml from 20.

The recoveries obtained with the addition of a known amount of pure riboflavin to samples of milk, food, feces and urine prior to and during the analytical procedure ranged from 87% to 105%.

#### DISCUSSION

The daily riboflavin intakes and excretions of 9 young college women were determined during three 5-day periods in the late spring of 1948. Three subjects, M.W., Z.H., and J.H., were observed throughout two full periods; D.J.M., for a part of two periods; and the 5 remaining subjects participated during only one period each.

The average daily riboflavin intake and the urinary output of each subject are give in table 1. The separate analysis of milk made it possible for individual needs and preferences to be met. This also accounts for the variation in the total riboflavin intake of different subjects during the same period.

The amount of milk consumed had a definite influence on the total riboflavin intake. Subject L.D. had a consistently lower riboflavin intake than the other subjects participating in period 3 because she did not drink milk. The daily milk consumption per girl ranged from none to three glasses, contributing an average of 140 to 933  $\mu\text{g/day}$ . The milk glasses used during period 3 were larger than those used previously. Also, the riboflavin content of the milk during this time was somewhat higher than in the preceding periods. This accounts for the fact that one serving of milk in period 3 contributed more riboflavin than did one serving in either of the

two other periods. The riboflavin content of the milk consumed during periods 1, 2, and 3 was 140, 120, and 200  $\mu\text{g}/100\text{ gm}$  of milk, respectively. These values fall within the range reported by Theophilus and Stromberg ('45) for daily samples of milk from Jersey and Holstein cows. However the range is wider, by a small margin, than that reported by Daniel and Norris ('44).

TABLE 1  
*Riboflavin intake with the per cent excretion in urine*

SUBJECT	AVERAGE DAILY INTAKE			AVERAGE DAILY EXCRETION	
	Food	Milk	Total	Total	Fraction
	$\mu\text{g}$	$\mu\text{g}$	$\mu\text{g}$	$\mu\text{g}$	%
Period 1 May 10-14, 1948					
M.W.	1349 $\pm$ 401	490 $\pm$ 120	1841 $\pm$ 534	718 $\pm$ 204	38 $\pm$ 8
Z.H.	1349 $\pm$ 401	455 $\pm$ 210	1806 $\pm$ 439	670 $\pm$ 253	38 $\pm$ 14
D.J.M.	1693 $\pm$ 397	700 $\pm$ 0	2393 $\pm$ 397	1134 $\pm$ 430	46 $\pm$ 11
Period 2 May 17-21, 1948					
M.W.	1131 $\pm$ 405	240 $\pm$ 54	1371 $\pm$ 446	478 $\pm$ 43	41 $\pm$ 9
Z.H.	1131 $\pm$ 405	240 $\pm$ 54	1371 $\pm$ 446	463 $\pm$ 72	36 $\pm$ 11
D.J.M.	1022 $\pm$ 374	675 $\pm$ 411	1447 $\pm$ 173	695 $\pm$ 53	48 $\pm$ 2
J.H.	1131 $\pm$ 405	300 $\pm$ 0	1431 $\pm$ 405	649 $\pm$ 108	51 $\pm$ 9
F.H.	1131 $\pm$ 405	300 $\pm$ 0	1431 $\pm$ 399	487 $\pm$ 21	39 $\pm$ 8
O.C.	1022 $\pm$ 374	225 $\pm$ 65	1247 $\pm$ 246	572 $\pm$ 144	51 $\pm$ 14
Period 3 May 24-28, 1948					
J.H.	1427 $\pm$ 462	560 $\pm$ 143	1987 $\pm$ 640	817 $\pm$ 195	44 $\pm$ 15
H.B.	1427 $\pm$ 462	700 $\pm$ 0	2127 $\pm$ 462	802 $\pm$ 295	40 $\pm$ 20
L.D.	1427 $\pm$ 462	140 $\pm$ 250	1567 $\pm$ 530	587 $\pm$ 157	43 $\pm$ 18
E.B.	1152 $\pm$ 266	933 $\pm$ 165	2469 $\pm$ 689	1157 $\pm$ 197	47 $\pm$ 16

The range of the average daily riboflavin intake (food plus milk) during periods 1, 2, and 3 was 1,806 to 2,393, 1,247 to 1,447, and 1,567 to 2,469  $\mu\text{g}$ , respectively. The total average daily intake during period 2 was somewhat lower than in the other two periods. This was associated with a decreased protein intake; the food protein (other than milk) averaged 65, 35 and 45 gm for the three periods. As a whole, on the days on which the riboflavin intake was less, the meats served were

fish, combination meat dishes or sandwiches. Only one subject had a negative nitrogen balance during this period. All the other subjects exhibited small nitrogen retentions. On this basis it was assumed that the previous diet, as well as the present one, was nutritionally adequate from the protein standpoint. The highest single daily intake of riboflavin from food alone occurred on the second day of period 3 when liver was the principal meat served. Tongue, custards, cheese, and foods with cream sauce also gave high riboflavin values.

The average daily urinary excretion of riboflavin ranged from 463 to 1,157  $\mu$ g, with a mean average of 710  $\mu$ g. This mean average agrees very well with the higher values reported by Brewer et al. ('46) and Strong et al. ('41) for young women on self-selected diets. The higher average excretion noted in the present study might be indicative of a higher intake; the intake in the above-mentioned studies was not determined. Subjects D.J.M. and E.B. of the present study consistently excreted more riboflavin in the urine than did the other subjects. These two, however, consumed a larger volume of milk than the others; consequently, the per cent of the daily intake excreted was not markedly higher.

The percentage of the average total daily intake excreted in the urine ranged from 36 to 51, with a mean average of 43%. Five subjects excreted from 46% to 51%, and 8, from 36% to 44%. These percentages are in close agreement with those reported by Everson et al. ('48) for 8 young women ingesting a daily diet supplying approximately 2.4 mg of riboflavin, although the average daily intake in the present study was lower; namely, 1.7 mg. The range in the Everson study was from 25% to 55%, with one subject excreting 55%; 6, from 33% to 43%; and one, 25%.

On the 5th day of period 2 a 5-mg supplement of riboflavin was given orally to each of 5 subjects, and the 24-hour urinary excretion was observed. The percentage of the 5-mg supplement returned (table 2) ranged from 24 to 44, with mean average of 34%. One subject returned 24% of the test dose; three, from 32% to 36%; and one, 44%. These values for

an average daily intake of 1,247 to 2,469  $\mu\text{g}$  are similar to those observed by Brewer et al. ('46) at levels of 790, 1,040, 1,260, 2,230 and 2,720  $\mu\text{g}$ . Their subjects were given a 3-mg test dose; they excreted 22, 30, 27, 31, and 56% in the 24 hours following the periods of controlled intake. It is interesting to note that Z.H. of the present study, who gave the lowest percentage excretion (36%) of the average total daily intake, also excreted the smallest percentage of the 5-mg supplement (24%).

The fecal output of riboflavin was determined on the three subjects of the first period and on 4 during the second period

TABLE 2  
*Urinary excretion of riboflavin following a 5-mg supplement*

SUBJECT	AVERAGE EXCRETION PRIOR TO SUPPLEMENT	EXCRETION FOLLOWING SUPPLEMENT	EXCRETION OF SUPPLEMENT
	$\mu\text{g}$	$\mu\text{g}$	%
M.W.	478	2,673	44
Z.H.	463	1,665	24
D.J.M.	695 <sup>1</sup>	2,288	32
J.H.	649	2,316	33
F.H.	487	2,268	36
Average	554	2,242	34

<sup>1</sup> Average excretion for two days only.

by administering a marker, as previously reported by Holt and Scoular ('48). The range of fecal output was greater during the first period than in the second; namely, from 515 to 954 and from 553 to 707  $\mu\text{g}/\text{day}$ , respectively. These values were obtained on diets which averaged from 1,247  $\mu\text{g}$  for the former to 1,447 for the latter. Only one subject (D.J.M.) participated during both of these periods. Her average output was less for the second period, 604  $\mu\text{g}$ , than the 885 excreted during the first period. As previously stated, the average protein intake was also lower during the second period. The 5-mg supplement was given the last day of the second period. Even so, there was no increase in the fecal output. It is apparent from these data that the supplement



was well absorbed. These fecal riboflavin values are higher than those reported by Hathaway and Lobb ('46) for women subjects on a synthetic diet supplying 1,090  $\mu\text{g}$  daily, and are lower than the excretion of the same subjects consuming a natural diet supplying 1,330  $\mu\text{g}$  daily. Z.H. of the present study, who had the highest output of fecal riboflavin, had diarrhea two days during the study. While it is known that some synthesis of riboflavin takes place in the intestines, it is not known to what extent this occurs. Consequently, a true balance cannot be determined, although it is evident that the total of the average fecal output and urinary excretion of riboflavin of these 5 subjects was in all instances less than the average riboflavin intake. Therefore both dietary and supplementary riboflavin were being absorbed.

#### SUMMARY

The riboflavin intake in food and excretion in urine of a group of 9 college women on self-selected diets were determined chemically. The fecal output of 5 of these subjects was also determined.

The average daily riboflavin intake, exclusive of the supplement given in one experimental period, ranged from 1,247 to 2,469  $\mu\text{g}$ .

The average daily urinary excretion of riboflavin on these intakes ranged from 36% to 51% of the intake.

Following a 5-mg supplement of riboflavin, one subject returned 24%; three returned 32% to 36%; and one returned 44%.

The average daily fecal output of 5 subjects ranged from 515 to 954  $\mu\text{g}$ , with a mean average of 682  $\mu\text{g}$ .

The total of the average daily fecal output and urinary excretion of riboflavin was less than the intake in all instances.

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# ARTIFICIAL ENRICHMENT OF WHITE RICE AS A SOLUTION TO ENDEMIC BERIBERI

## I. PRELIMINARY REPORT OF FIELD TRIALS<sup>1</sup>

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A complete beriberi survey in Bataan Province has been reported (Salcedo et al., '48) showing an incidence of symptoms of that disease in 12.77% of 12,400 people who were clinically examined. This survey was undertaken in contemplation of the introduction of artificially enriched rice in all municipalities on the Manila Bay side of the Province (68,000 people). This was inaugurated October 1, 1948, after the installation of machinery for quantitative mixing of "premix" with rice at each of 21 rice mills in the area. Labor and depreciation on this machinery, it is estimated, will add \$0.0008

<sup>1</sup>This study has been made possible by a grant from the Williams-Waterman Fund Committee of the Research Corporation, New York City, to the Department of Health of the Republic of the Philippines. The "premix" was donated by Hoffmann-LaRoche, Inc., Nutley, New Jersey, U.S.A. Equipment and supplies were authorized from funds allocated to the U. S. Public Health Service Rehabilitation Program in the Philippines in connection with the Philippine Rehabilitation Act of 1946 of the 79th Congress of the United States of America.

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<sup>5</sup>Rice Mill Engineering Consultant employed under a grant from the Williams-Waterman Fund.

per ganta (5 pounds) to the cost of rice. During the three months up to January 1, 1949, the enriched rice has proved acceptable to the population. The survey is to be repeated later to determine any change in incidence or in mortality from beriberi.

#### MATERIALS AND METHODS

Previous to the introduction of artificially enriched rice in Bataan Province, it was considered necessary to conduct preliminary field feeding trials to determine its "acceptability" in so far as color, taste, odor, palatability, and digestibility are concerned, as well as to determine any possible beneficial effects on general health and on existing peripheral neuritis and other symptoms of thiamine deficiency among the subjects tested. This was necessary in view of the disappointing experiences local public health officials had previously encountered with the introduction of brown rice, under-milled rice and certain varieties of parboiled rice. In the experiments cited below, as well as in the large-scale enrichment of rice being conducted in Bataan Province, we have used rice grains impregnated with thiamine, niacin and iron pyrophosphate, known as "premix," in such concentrations that when one part by weight of this "premix" is mixed with 200 parts by weight of white, polished rice (Furter et al., '46) the final mixture (now known as enriched rice) as delivered contains per pound 2.0 mg of thiamine, 15 mg of niacin, and 13 mg of iron. The nutrients to be used in a Philippine national enrichment program will be determined later, taking into consideration the results of present and future studies.

#### RESULTS AND OBSERVATIONS

Three sets of observations were made: I, among government employees; II, among children housed and fed in the Welfareville Institutions of the Social Welfare Commission; and III, among personnel of the Armed Forces of the Philippines.

### *I. Government employees*

A single 5-lb. trial quantity of enriched rice was given to each of 284 government employees and their respective families totalling, including the employees, 2,188 persons. All of these individuals consumed the enriched rice as a part of their usual daily menus and reported uniformly that it was found satisfactory and acceptable in all instances.

### *II. Welfareville institutions—enriched rice observations*

Two hundred eighty-seven children of both sexes and of different ages, ranging from three years to 18 years, were served enriched rice starting April 1, 1948. Another group of 287 children of similar sex and age distribution was served with the same amount of white milled rice, without "premix." The children in both groups each consumed daily 200 to 250 gm of rice in addition to the other components of their diet which, however, was generally deficient in thiamine when judged with respect to recommended daily allowances. Both groups were in fairly good health and showed no symptoms of thiamine deficiency at the start of or during the experiment. In Bataan Province it was found that clinical beriberi in children from two to 15 years of age is rare (Salcedo et al., '48). Before the project began, the children under observation were physically examined and their heights and weights were taken. Hemoglobin values and red cell counts were taken on 30 children in each group. On July 1, 1948, the same examinations were repeated to determine the effects of enriched rice on their health and nutrition.

*Results of observations three months after the start of the experiment —*

1. Average increase in height of the children: For the control group (no enriched rice given), 0.63 cm; for the group served with enriched rice, 0.79 cm.

2. The per cent of each group who increased in height by specified amounts is given in table 1.

3. Average gain in weight: For the control group (no enriched rice given), 0.62 kg; for the group served with enriched rice, 0.77 kg.

4. The per cent of each group who gained specified amounts in weight is given in table 2.

Comments. From the above data it may be inferred that the children served with enriched rice for a period of three months had increased in height and gained in weight more than those that were not served with enriched rice.

The above observations were continued for an additional period of 5 months, or a total of 8 months from the start, on 520 children, 260 serving as the experimental group (fed with enriched rice) and an equal number being used as controls.

TABLE 1

GROUPS UNDER OBSERVATION	NO IN- CREASE	INCREASE IN HEIGHT BETWEEN:			
		0.1- 1 cm	1.1- 2 cm	2.1- 3 cm	3.1- 4 cm
	%	%	%	%	%
1. Group served with enriched rice	7.66	66.50	24.00	1.84	0
2. Control group (no enriched rice)	11.84	63.76	22.64	1.76	0

TABLE 2

GROUPS UNDER OBSERVATION	NO GAIN	GAIN IN WEIGHT BETWEEN:			
		0.1- 1 kg	1.1- 2 kg	2.1- 3 kg	3.1- 4 kg
	%	%	%	%	%
1. Group served with enriched rice	9.90	63.36	21.78	4.45	0.51
2. Control group (no enriched rice)	9.42	76.63	11.43	2.52	0

Twenty-seven children from each group had to be dropped due to transfers and discharges from the institutions.

*Results of observations at end of eight months —*

1. Average increase in height of the children: for the control group (no enriched rice), 2.27 cm; for the group served with enriched rice, 2.34 cm.

2. The per cent of each group who increased in height by specified amounts is given in table 3.

3. Average gain in weight of the children: For the control group (no enriched rice), 1.43 kg; for the group served with enriched rice, 1.91 kg.

4. The per cent of each group who gained specified amounts in weight is given in table 4.

5. Average increase in hemoglobin %: For the control group (no enriched rice), 7.33%; for the group served with enriched rice, 13.83%.

6. Average increase in red cell count: For the control group (no enriched rice), 431,000 red cells; for the group served with enriched rice, 667,000 red cells.

Comments. From the above data it may be inferred that the children served with enriched rice for a period of 8 months had increased in height and gained in weight slightly more than those that were served with ordinary rice. It may

TABLE 3

GROUPS UNDER OBSERVATION	NO INCREASE	INCREASE IN HEIGHT BETWEEN:			
		0.1- 1 cm	1.1- 2 cm	2.1- 3 cm	3.1- 5 cm
1. Control group (no enriched rice)	% 4.61	% 15.76	% 24.23	% 27.69	% 27.71
2. Group served with enriched rice	% 2.30	% 17.69	% 21.15	% 30.38	% 28.48

TABLE 4

GROUPS UNDER OBSERVATION	DECREASED IN WEIGHT	NO GAIN IN WEIGHT	GAIN IN WEIGHT BETWEEN:			
			0.1-1 kg	1.1-2 kg	2.1-3 kg	3.1-5 kg
1. Control group	% 8.84	% 4.23	% 28.45	% 33.07	% 16.53	% 8.88
2. Group served with enriched rice	% 5.00	% 0.76	% 24.23	% 34.61	% 16.53	% 18.87

also be noted that more children among those served with enriched rice increased in height, from two to 5 cm, and gained in weight, from two to 5 kg. Fewer children among those served with enriched rice decreased in weight or gained no weight. It was also observed that enriched rice did not produce any unfavorable reactions among the children served with it.

### *III. Enriched rice feeding of Armed Forces personnel*

A clinical survey for beriberi was conducted among the personnel of certain units of the Armed Forces of the Philippines late in September, 1947, and repeated in July, 1948, as noted in table 5.

Enriched rice was issued to units 1 and 2 (table 5) and unenriched white rice to unit 3 from January 1 to mid-July, 1948. In addition, enriched rice was issued to 800 enlisted men of the First Infantry Training Battalion and to 150 civilian employees of the Ordnance Center. Thus, a total of 1,416 persons of the military establishment consumed enriched rice and were questioned and observed as to its acceptability and its effects. Clinical observations, however, were substantially confined to the personnel listed in table 5.

The use of enriched rice was not maintained consistently at the Ordnance Center, and in all three groups there was

TABLE 5  
*Experimental and control groups in armed forces*

UNIT	SEPT. 1947		JULY 1948		WEIGHTS <sup>1</sup>	
	Total men	No. with B <sub>1</sub> deficiency symptoms	Total men	No. with B <sub>1</sub> deficiency symptoms	In-creased (0.5-14 lb.)	De-creased (0.5-9 lb.)
1. Signal Service Bn. (exptl.)	130	21	93	0	60	15
2. Ordnance Center (exptl.)	220	67	143	25 <sup>2</sup>	51	81
3. Engineer Service Bn. (control)	116	13	83	30	40	27

<sup>1</sup> Weight changes are recorded only for those men who remained in the units throughout the period of experiment.

<sup>2</sup> Sixteen cases of this group had no symptoms during the first survey.

considerable movement of men to other camps. These disturbing factors interfered with the conduct of a rigorously controlled experiment, but certain of the results appear to be of interest.

*Findings.* A monthly check was made on all the men receiving the enriched rice, as to any reactions to it. All stated that there was no change in taste, color or palatability. There was a mild odor reminiscent of tikitiki. There was no increase in the incidence of gastrointestinal symptoms in the daily sick call. No untoward by-effects were attributed by the men to the enriched rice. All the men of both experimental and con-



trol groups were given the other components of their usual daily diets.

#### DISCUSSION

The Signal Service Battalion is a unit which showed the typical reaction due to constant feeding of enriched rice for 6 months, with general improvement in the physical appearance of the men. Sixty-five per cent of the men gained in weight, while 17% lost in weight. All of those with signs or symptoms of peripheral neuritis failed to show or complain of any further manifestations of this symptom-complex. Contrast this with a unit having a big work load, much movement of personnel, and irregular feeding with enriched rice, at the Ordnance Center. In this group there were 30% who increased in weight, 55% who lost in weight. Those cases which previously showed mild symptoms and signs were relieved but, although there was a big reduction in those with one or two signs or symptoms, 16 formerly showing no symptoms had developed symptoms of peripheral neuritis after 6 months. These 16 were among personnel who had been transferred to and from other ordnance units and those on cash rations permitting them to eat outside. Considering that their messes were practically identical with those of the Signal Battalion the different results indicate the effects of neglected use of enriched rice. The results shown in the Engineer Service Battalion (controls) indicated health improvement directly attributable to improved diet (caloric increase), but the increase in the number of cases with one or two signs or symptoms of polyneuritis indicated that the quality, at least the thiamine intake, was still subnormal. Contrast this with results in the Signal Service Battalion, where all cases with symptoms improved.

#### *Deaths from beriberi in Bataan Province*

Preliminary observations on the effect on beriberi mortality in Bataan of the introduction of artificially enriched



# NUTRITION STUDIES DURING PREGNANCY

## V. RELATION OF MATERNAL NUTRITION TO CONDITION OF INFANT AT BIRTH: STUDY OF SIBLINGS<sup>1</sup>

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### FIVE FIGURES

(Received for publication March 24, 1949)

### INTRODUCTION

Evidence that the nutrition of the human mother is important during pregnancy has become increasingly clear in recent years (Burke and Stuart, '48). Studies of the influence of maternal nutrition upon fetal growth and development, as well as investigation of its importance to the mother's health and course of pregnancy, have been and are being carried out in cooperation with the Boston Lying-in Hospital as a part of a larger research program on growth and development now in progress in the Department of Maternal and Child Health, School of Public Health, Harvard University.

Data on 216 women and their infants have already been published (Burke, Beal, Kirkwood and Stuart, '43; Burke, Harding and Stuart, '43). The present report deals with 68 younger infants born to 57 of these women. This group includes 57 second, 10 third siblings and one 4th sibling.

<sup>1</sup> This study was carried out under a grant from the General Education Board, Rockefeller Foundation, and is being completed under grants from the James Foundation and the Nutrition Foundation, New York City.

Data on this group of 68 siblings are compared with the published studies to learn whether in the two series a similar relationship exists between the mother's diet during pregnancy and the physical condition of her infant at birth.

Fifty-three pairs of first and second siblings<sup>2</sup> are presented for separate discussion because they offer a situation in which hereditary and environmental variables, in part, have been controlled. Of the 57 second siblings, all but 4 have been matched to a first sibling; 4 cases have been excluded because the mother at the first pregnancy gave birth to twins. By comparing these matched pairs of siblings it may be possible to learn whether the same mother on a like or a different diet tends to give birth to an infant in like or different physical condition. Since the kinds and amounts of food which a woman consumes are largely the result of long-established eating habits and the diets of the same mother tend to be similar in successive pregnancies, this attempt to compare the effect of maternal nutrition on matched siblings is difficult. In this paper only general ratings of the maternal diets during the latter part of pregnancy are used. No information was collected about the diets normally consumed by these women before or between pregnancies. Therefore, we do not know to what extent their food habits were changed during pregnancy except in that part of it under observation. Since no appreciable effort was made to change the food habits of these women during their pregnancies, it is likely that the dietary ratings given represent not only the diets of the women during pregnancy but, in the majority of cases, their long-time food habits. Hence, any association found between prenatal dietary rating and condition of infant at birth may be due either directly or indirectly to the diet during pregnancy, or to long-established food habits which tend to control a woman's nutritional state at the time she enters pregnancy, or to some combination of these two factors. It is possible that nutrition during the latter part of pregnancy

<sup>2</sup>First and second siblings refer to the order of children enrolled in the study and not necessarily to position in the family.

is a factor in certain relationships shown to exist, while long-established food habits may be important to both mother and fetus in other ways.

#### EXPERIMENTAL

##### *Method of collecting and evaluating prenatal diets*

The dietary histories taken from the 57 women going through one or more additional pregnancies were collected in the same manner as those in the published series (216 cases). Calculations were made based on these histories and the results evaluated in a manner already described in some detail in previous publications (Burke, Beal, Kirkwood and Stuart, '43; Burke, '47). It should be pointed out that the dietary histories give a picture of the *average* daily food intake of these women during the period studied. The ranges within the rating scale used absorb the majority of the inaccuracies which may remain in the dietary history, although our method of carefully cross checking all data obtained in the taking of the history reduces such errors to a minimum. The diets, as was the case previously, were rated in 5 categories.<sup>3</sup> This method of assigning a mean general dietary rating to the average daily intake of each woman during pregnancy places the women in a relatively exact relationship to each other on the basis of their average dietary intakes.

##### *Method of rating the physical condition of the infant at birth*

The pediatric ratings which describe the condition of the infants at birth and at the end of their two weeks' stay in the

<sup>3</sup> "Excellent," "good," "fair," "poor" and "very poor." The mean general dietary rating used in this paper was obtained in the following manner: numerical values were assigned to the ratings given each essential nutrient according to the range within which the calculated value fell: "Excellent" = 4; "good" = 3; "fair" = 2; "poor" = 1, and "very poor" = 0. The average caloric intake was also evaluated numerically. These numerical ratings were averaged and reconverted to the terminology representative of this average value of the diet. (See also footnote 5, p. 457.)

hospital nursery are based, as were those of the already published cases, upon the obstetrician's evaluation of the infant's condition at birth together with a routine physical examination by one of the pediatricians of the Growth Study Staff within 48 hours of birth and again before the infant's discharge from the hospital. The progress of the infant during his stay in the nursery was carefully recorded and any infant whose progress was not entirely satisfactory was seen frequently by one of the pediatricians on the Growth Study Staff. From this detailed information the pediatric ratings<sup>1</sup> for the 68 siblings were done independently by a pediatrician new to our staff.

*Consideration of original 216 cases with the addition of  
68 cases of siblings*

Figure 1 shows the relationship between the general dietary rating for pregnancy and the physical condition of the infant at birth when the 68 cases of siblings were added to the 216 published cases and the results depicted graphically, as in the original publication (Burke, Beal, Kirkwood and Stuart, '43, fig. 2, p. 580). In the 284 cases when the maternal diet was "excellent or good," 95% of the infants were in good or excellent physical condition at birth and only 5% were in either fair or poorest physical condition. In contrast, when the maternal diet was "poor to very poor" 65% of the infants

<sup>1</sup>An infant was called "excellent" ("superior" in the previously published series) when he had no physical count of any kind against him either at the birth examination or during his first two weeks of life; he was called "good" with only one or possibly two minor physical counts against him. An infant was called "poorest" when he was stillborn or died during the neonatal period, had a congenital malformation at birth, was premature (under 5 lb. at birth), or was "functionally immature" (physical development and performance of the infant considered below normal in some way other than weight and length alone). All other infants were termed in "fair" condition. This group includes some infants in a fair to good condition, others in a fair to poor condition, according to the pediatric findings. In interpreting these ratings, as in the case of the infants on whom findings have been published, those infants termed "good" are relatively close to those termed "excellent" in physical condition, while the group called "fair" represents a wider range.

were in the poorest physical condition at birth and 27% were in fair condition, while only 8% were in either good or excellent physical condition. Average birth weights and lengths of infants in each dietary category are included in figure 1 and in subsequent figures.

Figure 2 represents the 216 cases with the original "fair" diet group (149 cases) divided into 103 cases which comprise

284 CASES (216 FIRST SERIES + 68 SIBLINGS)

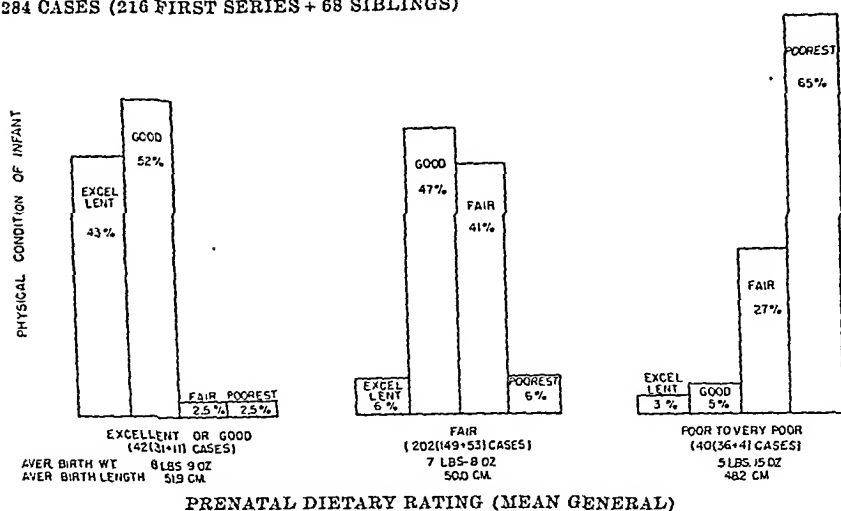


Fig. 1 Relationship of prenatal dietary rating (mean general) to the physical condition of the infant at birth. (Three maternal dietary groups, 284 cases.)

a somewhat narrower "fair" diet group; the remaining 46 cases are called "poor."<sup>5</sup> This division was made since further work led us to believe that the relationship between the condition of the fetus and a poor nutritional environment in the mother changes most abruptly as the diet becomes very poor. The lowest mean general dietary ratings are then

<sup>5</sup>The original "fair" diet group contained all mean general dietary ratings from 2.7 to 1.1 inclusive. In dividing this rather wide "fair" diet group, the new "fair" diet group was made to include only mean general dietary ratings from 2.7 to 1.8 inclusive. The ratings from 1.7 to 1.1 inclusive are termed "poor." The lowest mean general dietary ratings, as was the case previously with those under 1.1, are called "very poor." Mean general dietary ratings from 2.8 to 3.2 inclusive are termed "good" and of 3.2 or more "excellent."

termed "very poor" and represent an extremely deficient dietary even in the minds of the most conservative. The division into 4 dietary groups, used in figure 2, shows more clearly the consistent trend toward lower physical ratings of infants with lower maternal dietary ratings, and particularly the abrupt increase in the percentage of poorest infants as the diet changes from "poor" to "very poor." It is evident that a "very poor" maternal diet is associated with an infant in the

## 216 CASES (FIRST SERIES)

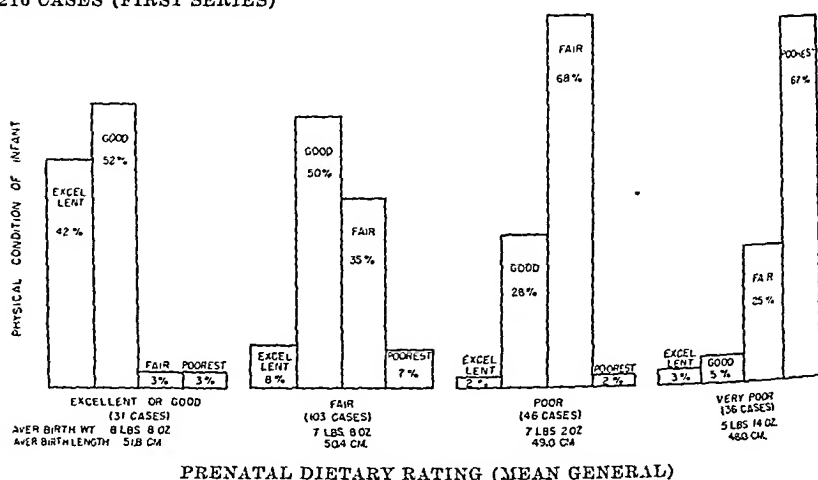


Fig. 2 Relationship of prenatal dietary rating (mean general) to the physical condition of the infant at birth. (Four maternal dietary groups, 216 cases, first series.)

poorest physical condition at birth, while an "excellent or good" diet is associated with an infant in excellent or good condition at birth. Furthermore, figure 2 illustrates the consistent shift in physical condition of the infant with each change in either direction in the mean general prenatal dietary rating.

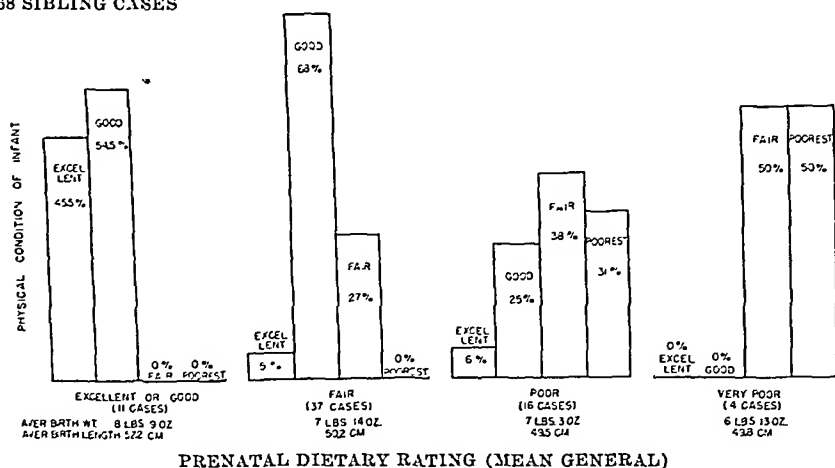
Figure 3 presents the data on the 68 siblings in like manner. An essentially similar graph results, except that the "poor" diet group contains a considerably higher incidence of poorest infants than was found in the original 216 cases.



*Relation of average birth weights and birth lengths of infants to prenatal dietary ratings (mean general)*

The average birth weights and lengths of the infants in the various maternal dietary categories are given in each figure shown. In every instance the average birth lengths and weights decrease as the maternal dietary ratings become poorer. The constancy of this relationship in every grouping of cases is impressive.

68 SIBLING CASES



PRENATAL DIETARY RATING (MEAN GENERAL)

Fig. 3 Relationship of prenatal dietary rating (mean general) to the physical condition of the infant at birth. (Four maternal dietary groups, 68 sibling cases.)

Since physical condition of infant and birth weight and length are to some extent related, and physical condition of infant at birth and maternal dietary ratings are closely related, the question arises: Are birth weight and birth length more closely associated with physical condition of infant at birth than with maternal dietary rating? Table 1 attempts to answer this question by listing average birth weights and lengths of infants according to the prenatal dietary ratings and according to the physical condition of the infants at birth. Although a study of this table demonstrates a relationship

TABLE 1

*Average birth weights and birth lengths of infants (284) grouped according to prenatal dietary rating (mean general) and according to physical condition of infant at birth*

PHYSICAL CONDITION OF INFANT AT BIRTH	AVERAGE BIRTH WEIGHT AVERAGE BIRTH LENGTH	PRENATAL DIETARY RATING — MEAN GENERAL					Total no. cases
		Excellent	Good	Fair	Poor	Very poor	
Excellent	(10) <sup>1</sup>	(8)	(10)	(2)	(1)		31
Weight lb. oz.	8-10	8-9	7-10	8-2	6-10		
Length cm	52.2	51.2	50.5	52.2	50.3		
Good	(3)	(19)	(77)	(17)	(2)		118
Weight lb. oz.	9-7	8-6	7-13	7-3	6-8		
Length cm	52.6	51.9	50.5	48.9	47.6		
Fair	(0)	(1)	(46)	(37)	(11)		95
Weight lb. oz.	...	7-4	7-8	7-1	6-8		
Length cm	...	51.2	50.3	48.9 <sup>2</sup>	48.8		
Poorest	(1)	(0)	(7)	(6)	(25)		40
Weight lb. oz.	8-11	...	6-10	7-3	5-10 <sup>3</sup>		
Length cm	52.6	...	48.6	51.3	47.8 <sup>2</sup>		
Total no. cases	14	28	140	62	40		284 (216 + 68)

<sup>1</sup> Numbers in upper left hand corners = number of cases.

<sup>2</sup> Four cases no birth lengths.

<sup>3</sup> One case no birth weight.

between physical condition at birth and birth weight and length, a closer relationship is shown between maternal dietary rating for pregnancy and birth weight and length of infant.

### *Comparison of 53 matched pairs of siblings*

Figure 4 depicts the relationship between the maternal dietary ratings and the physical condition of the first siblings

#### 53 FIRST SIBLINGS

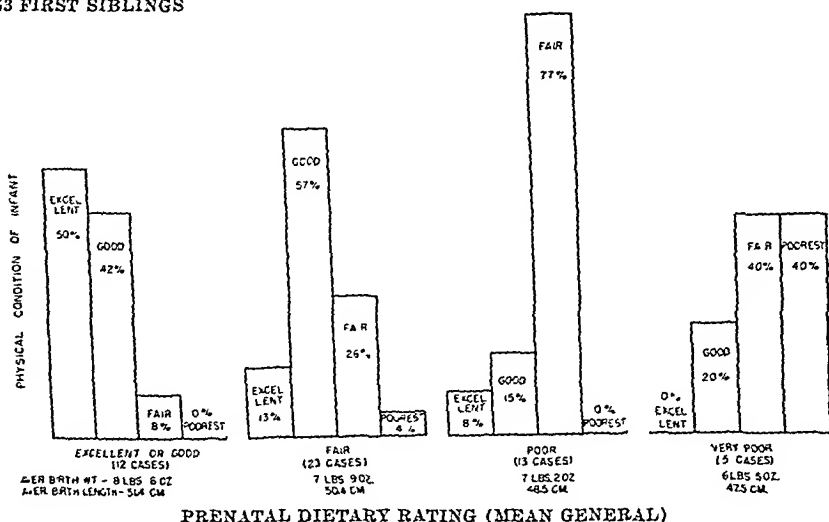


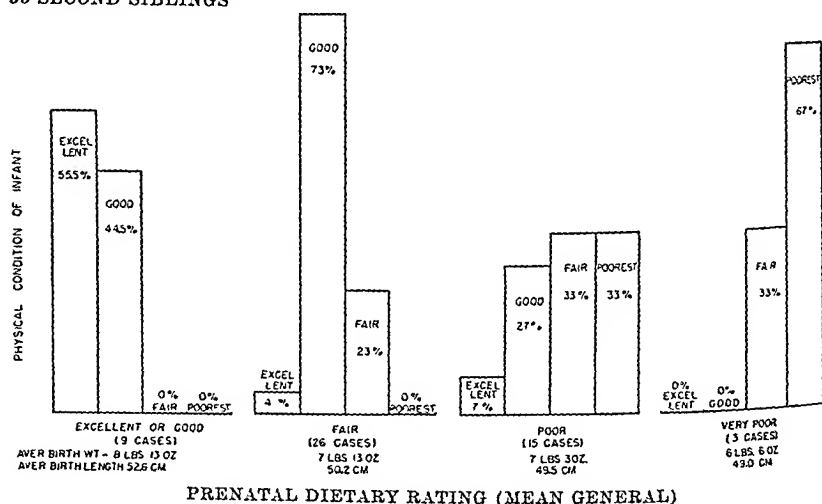
Fig. 4 Relationship of prenatal dietary rating (mean general) to the physical condition of the infant at birth. (Four maternal dietary groups, 53 first siblings.)

of the 53 matched cases (taken from the 216 original cases). Figure 5 shows the same relationship for the second siblings, younger brothers or sisters of the first siblings. These figures illustrate again in a striking manner the consistent shift in the physical condition of the infant with each change in either direction in the mean general prenatal dietary rating.

The relationship between the prenatal dietary ratings of the 53 mothers during their first and second pregnancies was studied. Thirty of the 53 women had the same dietary ratings during both pregnancies. Six of the mothers improved their

dietary ratings by one category and 5 improved them by two categories. On the other hand, 7 of the mothers had dietary ratings during their second pregnancies which were poorer by one category than those during the first, while 5 showed ratings which were worse by two categories. The group as a whole remained at about the same dietary level during their two pregnancies. It is well known that to change an individual's food habits is time-consuming and costly. Apparently greater motivation and more education are required

## 53 SECOND SIBLINGS



PRENATAL DIETARY RATING (MEAN GENERAL)

Fig. 5 Relationship of prenatal dietary rating (mean general) to the physical condition of the infant at birth. (Four maternal dietary groups, 53 second siblings matched to 53 first siblings.)

than were given to this group of women. It is unfortunate for this study that the diets of these women tended to remain the same or changed only slightly during their successive pregnancies.

The physical ratings of the first and second siblings of the 53 mothers are shown in table 2. These mothers tended to produce pairs of offspring in the same condition during their successive pregnancies. It should be noted that these 53 mothers, who enrolled two or more of their children in the

Growth Study, produced better babies than did the remainder of the 216 mothers. There were neither stillbirths nor neonatal deaths in the 106 children involved here.

Table 3 lists all the poorest first and second siblings in the 53 matched pairs, and includes an explanation of their physical ratings. It compares each infant with his sibling and with the dietary ratings of their mother during her two pregnancies. It is of interest that of the 12 poorest infants 4 comprised two pairs of siblings. Of the remaining 8 poorest siblings, 5 are paired with a brother or sister in fair condition. In only three instances was a poorest sibling paired with an infant in good or excellent physical condition.

TABLE 2  
*Physical ratings at birth on first and second siblings*

RATINGS ON SECOND SIBLINGS	RATINGS ON FIRST SIBLINGS				Total
	Exc.	Good	Fair	Poorest	
Excellent	1	5	1	0	7
Good	7	12	6	2	27
Fair	1	3	7	1	12
Poorest	1	1	3	2	7
Total	10	21	17	5	53

Table 4 shows the change in the condition at birth of the second sibling from that of his older brother or sister with the change in the mean general prenatal dietary rating. The diet was considered to have changed for better or worse if it was shifted one or more dietary categories (i.e., from "good" to "excellent," etc.). Eleven mothers improved their diets during their second pregnancies. Six of these produced second infants which were in better condition than the first, while only three produced second babies in worse condition. Among 12 mothers whose diets were worse during their second pregnancies, 5 produced second babies in worse condition, while only two produced better infants.

It has been pointed out previously that there were 10 mothers whose diets had changed by two categories. It is of

some interest to see what happened to the respective babies. There were three mothers whose diets shifted from fair to excellent: in these cases the condition of the babies changed from good to excellent. There were two mothers whose diets changed from very poor to fair: in one of these cases the condition of the second sibling improved from poor to fair, while in the other the condition of both babies was good. There were 5 mothers whose diets during their second preg-

TABLE 3

*Comparison of all poorest first and second siblings in the 53 matched pairs with their sibling and with the dietary ratings of the mother during the two pregnancies*

CASE NO.		PHYSICAL CONDITION OF INFANT AT BIRTH	PRENATAL DIETARY RATING (mean general)
First sibling	Second sibling		
50	130	Fair Poorest — functionally immature	Very poor Poor
32	131	Fair Poorest — hemorrhagic disease of newborn	Poor Poor
56	145	Fair Poorest — hemorrhagic disease of newborn	Poor Poor
24	170	Excellent Poorest — congenital malformation of ribs	Poor Poor
250	270	Good Poorest — club foot, hammer toe	Fair Poor
116	214	Poorest — bifid uvula Poorest — cleft palate (feeble-minded at 1 yr.)	Fair Very poor
158	228	Poorest — premature (3 lb. 14 oz.) Poorest — premature (3 lb. 15 oz.)	Very poor Very poor
81	239	Poorest — congenital heart (heart murmur prominent during first year; rapidly disappeared thereafter) Fair	Excellent Good
231	285	Poorest — cleft palate (feeble-minded at 1 yr.) Fair	Very poor Fair
191	305	Poorest — premature (3 lb. 6 oz.), inguinal hernia Good	Fair Fair

nancies were worse by two categories than those during the first pregnancies. For 4 of these the dietary change was from excellent to fair. The condition of the infant in two of these cases deteriorated, it remained the same in one case, and the second infant was better than the first in the other case. There was one mother whose diet shifted from fair to very poor and both of her infants were rated poorest at birth.

Improvement of the infant's condition with improvement of the maternal diet and deterioration of the infant's condition with deterioration of the maternal diet appear in this series where each of the 53 mothers was observed through two pregnancies, but the changes are not sufficiently striking

TABLE 4

*Change in condition of infant with change in maternal dietary rating  
(mean general)*

SECOND MATERNAL DIETARY RATINGS	SECOND SIBLINGS			Total
	Better	Same	Worse	
Better	6	2	3	11
Same	7	15	8	30
Worse	2	5	5	12
Total	15	22	16	53

to be statistically significant. Nevertheless, this particular series of matched siblings is unique since it has been possible to use each mother as her own control and thus to eliminate to some extent certain of the extraneous factors which influence the condition of babies at birth.

#### SUMMARY AND CONCLUSIONS

A study of the physical condition at birth of 68 siblings (57 second, 10 third siblings and one fourth sibling) born to 57 of the women studied in a previously published series of cases has been made in relation to the mean general dietary rating for the latter part of these subsequent pregnancies.

1. In the 284 cases when the maternal diet was "excellent or good," 95% of the infants were in good or excellent physi-

cal condition at birth and only 5% were in either fair or poorest physical condition. In contrast, when the maternal diet was "poor to very poor" 65% of the infants were in the poorest physical condition at birth and 27% were in fair condition, while only 8% were in either good or excellent physical condition.

2. The "fair" diet group was divided into two groups, thus giving 4 maternal dietary groupings: (1) "excellent or good"; (2) "fair"; (3) "poor"; and (4) "very poor." This has helped to make clear that a "very poor" maternal diet is associated with an infant in the poorest physical condition at birth, while an "excellent or good" diet is associated with an infant in excellent or good condition at birth. The consistent shift in physical condition of the infant with each change in dietary rating in either direction is impressive.

3. The statement still remains true that all of the stillborn infants, all of the neonatal deaths but one, all of the premature infants but one (a neonatal death), all of the functionally immature infants, and most of the congenital defects were found in the group of infants born to mothers with "poor" or "very poor" prenatal diets. Since no major effort was made to change the dietary habits of these women, it is probable that in the majority of the cases the prenatal dietary rating was representative also of long-time food habits.

4. The average birth weight and birth length of the infant decreased as the maternal dietary rating became poorer. The constancy of this relationship is impressive. While a relationship exists between physical condition of infant and birth weight and length, a closer relationship is shown between maternal dietary rating for pregnancy and birth weight and length of infant.

5. Data were presented on 53 matched pairs of siblings. Improvement of the infant's condition with improvement of the maternal diet and deterioration of the infant's condition with deterioration of the maternal diet appeared in this series where each of the 53 mothers was observed through two pregnancies. While the changes were not sufficiently striking to



be statistically significant, it is felt that this series of matched siblings is unique since it has been possible to use each mother as her own control and thus in part minimize hereditary and environmental variables which influence the condition of babies at birth. Similar studies of women through successive pregnancies may help further to clarify the relationship between prenatal diet and the condition of the infant at birth.

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# FOLIC ACID STUDIES IN THE MINK AND FOX<sup>1</sup>

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## TWO FIGURES

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Previous publications from this laboratory have shown that mink and foxes require a dietary source of folic acid (Schaefer et al., '46, '47). Later it was shown that an unidentified factor present in a 60% methanol extract of fresh liver is also needed (Schaefer, Whitehair and Elvehjem, '48). Since this extract was found to contain only 2.0 to 2.3  $\mu$ g of folic acid per milliliter,<sup>3</sup> and in view of the similarity of syndrome of the two deficiency states, we undertook a study of the interrelationship between these two dietary essentials. An earlier observation on the inability of the fox to use the conjugated forms of folic acid was studied further through the use of crystalline materials.

## EXPERIMENTAL AND RESULTS

Four fox pups and 29 mink kits were placed on a purified basal ration which had the following composition in grams: sucrose 66, casein<sup>4</sup> 19, salts IV (Phillips and Hart, '35) 4,

<sup>1</sup>Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. These studies were supported by project 614 of the Wisconsin Agricultural Experiment Station, and by a grant from the Borden Company, New York, N. Y.

A portion of this work was reported briefly in *Fed. Proc.*, 8: 399, 1949.

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<sup>3</sup>We are indebted to O. E. Olson for the microbiological assay of folic acid in this material.

<sup>4</sup>Smaco vitamin test.

and cottonseed oil 11. Each 100 gm ration was supplemented with 0.2 mg thiamine HCl, 0.2 mg pyridoxine HCl, 0.4 mg riboflavin, 1.5 mg Ca pantothenate, 4.0 mg nicotinic acid, 100 mg choline, 25 mg *i*-inositol, 50 mg *p*-aminobenzoic acid, 0.5 mg 2-methyl 1-4 naphthoquinone, and 0.025 mg biotin. Haliver oil fortified with  $\alpha$ -tocopherol acetate and vitamin D<sub>3</sub> was added so that each 100 gm ration contained 4 mg  $\alpha$ -tocopherol, 1,200 I. U. vitamin A and 120 I. U. vitamin D<sub>3</sub>. The care and handling of the animals were the same as in previous experiments.

### *Fox studies*

After receiving the basal ration for 13 weeks, a loss of body weight (fig. 1) and a decrease in hemoglobin content of the blood were noted in fox 52. Thirty milliliters of a commercial refined liver extract<sup>5</sup> were given parenterally in doses of 3 ml every third day. No response in body weight or hemoglobin resulted, and the ration was supplemented with 0.1 mg folic acid per 100 gm ration. Body weight increased 1.26 kg in 10 weeks and the hemoglobin increased 2.5 gm% in the same period of time.

When fox 58 (fig. 1) had been on the basal ration 12 weeks a decline in body weight occurred, and three weeks later anemia and anorexia developed. Five milliliters of the methanol extract of fresh liver (1 ml = 10 gm liver) was administered orally and the anorexia was corrected. The methanol extract was then included in the ration at a level of 1% (1 ml per 100 gm ration), and an immediate response in body weight followed. The hemoglobin, which had varied between 12.8 and 12.1 gm%, increased to 15.5 when the methanol extract was given.

After foxes 62 and 63 (fig. 1) had received the basal ration for 18 and 14 weeks respectively, the syndrome of folic acid deficiency developed. Six milligrams of folic acid as the heptaglutamate were injected intravenously (2 mg every

<sup>5</sup> Sharp and Dohme No. 2505, 15 U.S.P. antipernicious anemia units per milliliter.

other day) into the latter animal, but loss of body weight and hemoglobin continued. Two milligrams of folic acid were given by intravenous injection and 18 days later an additional 2 mg were administered. A prompt increase in body weight and hemoglobin content of the blood resulted. In fox 62, 2.5 mg folic acid as the heptaglutamate were injected intravenously, and when it was evident that no response

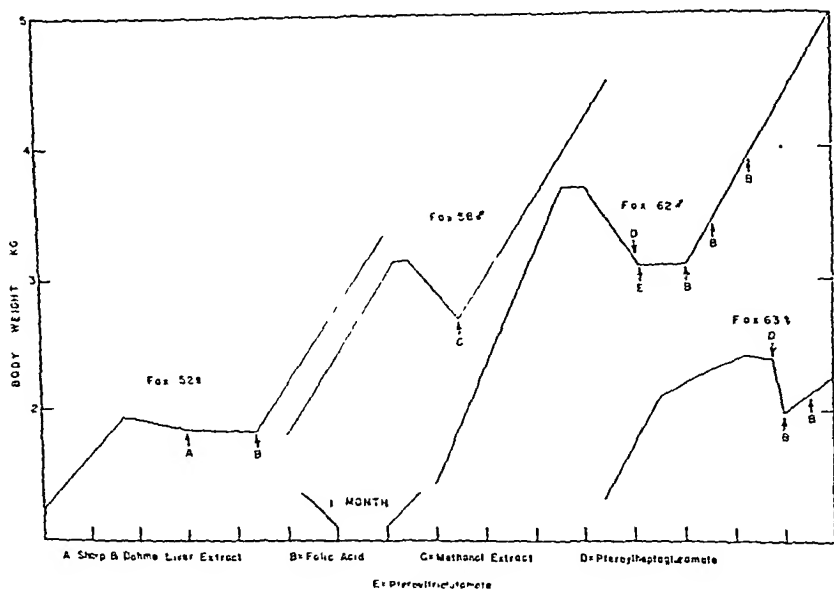


Fig. 1 Growth curves for foxes receiving the basal diet and the basal diet supplemented with folic acid and liver extracts.

would be obtained 5 mg of pteroyl-triglutamic acid<sup>6</sup> were also administered. Hemoglobin levels remained between 10.7 and 11.5 gm%, and no increase in body weight was noted. Twenty-six days later 2 mg folic acid were given parenterally, and two more injections at the same level were given at two-week intervals. Three months after receiving the first injection of folic acid, body weight had increased 2.9 kg, but hemoglobin remained at relatively the same level.

<sup>6</sup> "Teroplerin."

*Mink studies*

Mink 74 (table 1), receiving the folic acid-"free" ration, grew normally for 7 weeks and then failed to grow. When 1% methanol extract was added to the diet, body weight increased 325 gm in 8 weeks and the hemoglobin increased 3.6 gm%. A second decline in body weight and hemoglobin then occurred even though the extract was continued. Five milligrams of folic acid administered parenterally produced no improvement and the animal died. In light of previous work, death was probably due to a lack of the residue factor (Schaefer, Tove, Whitehair and Elvehjem, '48).

Mink 175 and 177 (table 1), after receiving the basal diet for 8 weeks, lost 500 and 430 gm, respectively. The former animal was given 1% of the methanol extract and body weight increased 310 gm in three weeks. The same level of extract was injected intramuscularly (based on food consumption of the previous day) into mink 177, and in three weeks body weight increased 300 gm. Two animals, 109 and 107, were given the basal ration supplemented with 1 ml methanol extract per 100 gm ration. Mink 109 appeared normal in both body weight and hemoglobin after 7 months on experiment. Mink 107 lost weight after receiving this diet for 16 weeks, but the administration of folic acid either in a single dose of 1 mg or the addition of 0.1 mg per 100 gm ration had no effect. A response was obtained, however, upon the addition of 4% residue to the ration.

Mink 134 and 157 exhibited deficiency symptoms after receiving the basal ration for 4 and 8 weeks, respectively. Parenteral administration of a commercial liver concentrate<sup>1</sup> equivalent to five-tenths or one antipernicious anemia unit in the former animal and five-tenths unit in the latter, had no effect. However, when the methanol extract was given, a response in body weight resulted. The daily injection of 1  $\mu$ g vitamin B<sub>12</sub> for 5 days was ineffective in alleviating symptoms of a lack of the methanol extract factor exhibited by

<sup>1</sup> Eli Lilly and Company. Reticulogen, 20 U.S.P. antipernicious anemia units per milliliter.

mink 178. Vitamin B<sub>12</sub> also failed to elicit a response in mink 142 receiving 4% residue in addition to the basal ration supplemented with folic acid.

TABLE 1

*Effect of various supplements on growth of mink receiving the basal ration*

MINK NO.	RATION	DAYS ON EXPERIMENT	BODY WEIGHT	SUPPLEMENT	DAYS	BODY WEIGHT
			gm			gm
74	Basal	50	595	1% MeOH ext.	59	920
175	Basal	56	770	1% MeOH ext.	21	1080
177	Basal	59	865	1% MeOH ext.	21	1165
109	Basal + 1% MeOH ext.	182	1170	..	..	....
107	Basal + 1% MeOH ext.	114	850	1 mg folic acid <sup>1</sup>	12	835
				0.1 mg folic acid/100 gm	42	775
134	Basal	29	695	0.025 ml Reticulogen/day <sup>2</sup>	27	760
				0.05 ml Reticulogen/day	21	740
				1% MeOH ext.	14	695
				2% MeOH ext.	63	1135
157	Basal	63	810	0.025 ml Reticulogen/day	21	785
				1% MeOH ext.	77	1250
178	Basal + 0.1 mg folic acid	86	1030	1 µg B <sub>12</sub> /day <sup>3</sup>	5	910
				1% MeOH ext.	6	970
				1% MeOH ext.	42	1215
142	Basal + 0.1 mg folic acid + 4% residue	69	825	1 µg B <sub>12</sub> /day <sup>3</sup>	4	740
				1% MeOH ext.	25	1020

<sup>1</sup> Single dose injected intramuscularly.

<sup>2</sup> Refined liver extract containing 20 U.S.P. antipernicious anemia units per milliliter, injected intramuscularly.

<sup>3</sup> Given daily by intramuscular injection.

The effect of a crude antagonist of folic acid (7-methyl folic acid) was studied with mink 76, 82 and 112 (fig. 2). Three-tenths per cent of the antagonist was added to the basal diet supplemented with 0.1 mg folic acid per 100 gm ration. One per cent methanol extract was also added to the ration of mink 76. Immediate loss of body weight occurred in animals 82 and 112, and when 1% methanol extract was added to the ration an increase in body weight resulted. The response was not complete and a second decline occurred.

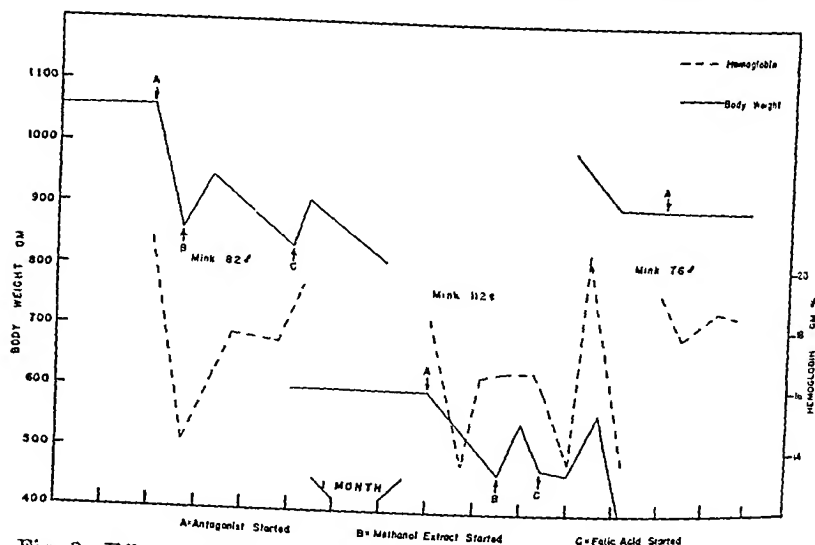


Fig. 2 Effect of folic acid antagonist on body weight and hemoglobin content of blood of mink.

The folic acid was increased to 0.2 mg per 100 gm ration and a second response was obtained, although the body weight did not return to a normal value. No change in body weight was observed in mink 76, which received the extract from the start.

There was a marked decrease in hemoglobin in animals 82 and 112 upon the addition of the antagonist. In the former animal the addition of the extract resulted in an increase of 3.7 gm%. When body weight declined the second time the hemoglobin also fell slightly, but rose again when the folic acid



content of the ration was increased. In mink 112, however, hemoglobin rose spontaneously before the extract was given, and the addition of the methanol extract produced no further increase. After the folic acid content of the ration was raised, the hemoglobin level declined but rose again as body weight increased. In the third animal, mink 76, the hemoglobin level remained quite normal.

The folic acid content of the blood, urine and feces of mink receiving the basal ration supplemented with either folic acid or the methanol extract or both, was determined. The assay used was the turbidimetric method of Luckey, Briggs and Elvehjem ('44) with *Streptococcus faecalis* (American Type Culture Collection No. 8043). Since it had been observed<sup>8</sup> that the incubation of blood with buffer at pH 7 resulted in an increase of free folic acid as great as that obtained when enzyme preparations were added, the former procedure was adopted. Accordingly, blood samples were prepared in the following manner.<sup>9</sup>

Three or 4 ml oxalated blood obtained by cardiac puncture were added to 5 ml pH 7.0 buffer and incubated under toluene for 4 hours at 37°C. A crystal of meta phosphoric acid was added and the sample placed in a boiling water bath for three minutes. It was then cooled, neutralized, diluted to a suitable volume and filtered.

Twenty-four-hour samples of urine and feces were collected by placing the animals in metabolism cages. Food consumption records were kept. The urine was collected in dark bottles containing toluene, diluted to a suitable volume and filtered for assay. Feces were homogenized and allowed to autolyze under toluene 72 hours at 37°C., and then autoclaved for 15 minutes at 15 lb. pressure. Folic acid was released enzymatically by incubating the sample first with a hog pancreas preparation and then with a hog kidney enzyme prepa-

<sup>8</sup> Olson, O. E., E. E. C. Fager, R. H. Burris and C. A. Elvehjem, unpublished data.

<sup>9</sup> This procedure was developed by O. E. Olson and modified by A. Sreenivasan and A. E. Harper at this laboratory.

ration. The general procedure of Sreenivasan, Harper and Elvehjem ('49) was followed.

The results of these assays are presented in table 2. The addition of 1% methanol extract to the basal ration produced the same increase in the folic acid content of blood, urine and feces as did supplementing the diet with 0.1 mg folic acid per 100 gm ration. While the methanol extract did not increase the blood folic acid of animals receiving 0.1 mg folic acid per 100 gm ration, it did produce an increase in the urine and fecal levels.

TABLE 2

*Effect of methanol extract of fresh liver on folic acid content of blood, urine and feces of mink*

RATION	SERIES 1				SERIES 2			
	No. mink	Blood folic acid	No. mink	Urine folic acid $\mu\text{g/gm}$ ration consumed	No. mink	Urine folic acid $\mu\text{g/gm}$ ration consumed	No. mink	Fecal folic acid $\mu\text{g/gm}$ ration consumed
		$\text{m}\mu\text{g/ml}$						
Basal	4	< 1.95	2	1.45	.	....	2	< 0.19
Basal + 0.025 mg folic acid per 100 gm ration	3	< 1.80	4	2.60	5	3.03	1	0.27
Basal + 0.1 mg folic acid per 100 gm ration	7	3.80	5	2.81	7	3.70	2	0.82
Basal + 1% MeOH ext.	3	3.65	1	2.06	4	5.98	1	0.99
Basal + 0.1 mg folic acid per 100 gm ration + 1% MeOH ext.	3	3.32	1	4.60	2	4.09	1	3.88

## DISCUSSION

Since the methanol extract, which contains only 2  $\mu$ g folic acid per milliliter, will replace folic acid in the ration of the mink and the fox and reverses the crude folic acid antagonist in the mink, it becomes apparent that the methanol extract contains a factor which has a sparing action on folic acid. Further support is given this hypothesis by the fact that the blood, urine and fecal levels of folic acid increased when the methanol extract was added to the ration.

Our results confirm the observations of Cartwright et al. ('48) and Cunha et al. ('48), with pigs, that antipernicious anemia liver extracts fail to replace folic acid. The inactivity of these commercial liver extracts and of crystalline B<sub>12</sub> indicates that the methanol extract factor is different from vitamin B<sub>12</sub>.

Day and Totter ('48) have reported that only small amounts of the folic acid fed to monkeys are recovered in the urine. A similar observation with respect to pterioic acid in humans was made by Franklin, Stokstad and Jukes ('47). The results of the folic acid assays in our experiments reveal the same situation applying to the mink.

The earlier observation that folic acid conjugate (as yeast) failed to replace folic acid for the fox has been confirmed by the use of the crystalline compound. Since the conjugate was injected intravenously, it would appear that the animal is incapable of hydrolyzing the heptaglutamate compound and that the inactivity is not due merely to lack of absorption of a large molecule or lack of hydrolysis within the intestinal tract. Preliminary results indicate that pteroyl-triglutamic acid is also inactive.

## SUMMARY

1. A methanol extract of liver contains a factor which has a sparing action on the dietary folic acid requirement of the mink.

2. The effect obtained with the methanol extract is not obtained with vitamin B<sub>12</sub> under our experimental conditions.

3. Folic acid conjugates are incapable of replacing folic acid for the fox.

#### ACKNOWLEDGMENTS

We wish to acknowledge our indebtedness to Merck and Company, Rahway, New Jersey, for the crystalline vitamins, including vitamin B<sub>12</sub>; to Lederle Laboratories, Inc., Pearl River, New York, for the synthetic folic acid, "Teropterin" and crude antagonist of folic acid; and to Parke Davis and Company, Detroit, Michigan, for the folic acid conjugate.

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# THE IRON METABOLISM OF YOUNG WOMEN ON TWO LEVELS OF INTAKE

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Additional information is needed regarding the iron requirement of women. The National Research Council ('48) recommendation for an intake of 12 mg per day was arrived at by making an estimate of the requirement and adding an amount supplying a factor of safety. There is little likelihood that further work will demonstrate that this allowance should be raised, but more work needs to be done to test lower levels of intake. A study of the iron metabolism of young women carried out in this laboratory included, along with an investigation of the absorption of iron from beef (Johnston, Frenchman and Boroughs, '48) which has already been reported, a study of the adequacy of two levels of iron intake. One level was much lower than the recommendation of the National Research Council, and one approached it.

## EXPERIMENTAL

Five young women 18 years of age were maintained for 10 weeks on a controlled diet. The total period was divided into a two-week adjustment period and two experimental periods each 4 weeks long. During the adjustment period and the first experimental period the diet contained 7 mg of iron a day, and during the second experimental period it contained 10.4 mg per day. The basal diet for all 10 weeks was

of a dietary pattern considered adequate by nutritionists; it included in each day one serving of a flesh food, one egg, one quart of milk, two servings of vegetables besides potatoes, and two or more servings of fruit. All the bread was made from an especially prepared unenriched flour.<sup>1</sup> To avoid a high phytic acid content the diet contained no whole cereals, shelled beans, nuts or cocoa and only one chocolate flavored food a week. The only change made in the diet when the iron content was increased to 10.4 mg was the addition of two 100-gm patties of ground beef made up of one-third fat and two-thirds lean meat; one was given in the breakfast and the other in the lunch. This resulted in a diet containing meat in every meal.

Composites of food were made each week for the entire 10 weeks; composites of stools were made for each week except for the two preliminary weeks; composites of urine were made for only a 4-day period because the excretion of iron in the urine is constant for a given individual and is so small as to be of little importance; composites of the menses were made for each period occurring during the 10 weeks. The method of collection, the preparation for analysis and the analyses of the foods and stools were described in the previous paper (Johnston, Frenchman and Boroughs, '48). The urines were treated with concentrated HCl and bottled for analysis at a later date. The menses were collected on tampon-type pads and the pads were dropped into redistilled water and let stand for several hours, after which enough HCl was added to make a final concentration of 10% and the whole heated until the pads disintegrated. A correction was made for the iron content of the pads. The urine and menses were analyzed in the same way as the foods and stools: they were ashed with sulphuric and nitric acids, and iron determined by the thiocyanate method of Stugart ('31).

<sup>1</sup> The flour was especially prepared for this project without enrichment by the Pillsbury Mills, Inc.

## RESULTS

Retentions were calculated by subtracting the iron content of the urine and stools from the iron of the foods. Mean iron retentions per day for 4 weeks on an intake of approximately 7 mg of iron were 0.26, 0.43, 0.61, 0.64 and 1.33 mg (table 1) for the 5 subjects. After beef was added to raise the intake to approximately 10.4 mg, the retentions increased to 1.77, 1.79, 1.80, 2.37 and 2.53 per day (table 2).

TABLE 1

*Retentions of iron on an intake of approximately 7 mg a day for 4 weeks*

SUBJECT	INTAKE	EXCRETION		RETENTION
		Urine	Feces	
	<i>mg/day</i>	<i>mg/day</i>	<i>mg/day</i>	<i>mg/day</i>
A	6.90	0.06	6.20	0.64
B	7.00	0.06	6.68	0.26
C	7.08	0.07	6.58	0.43
D	6.53	0.08	5.84	0.61
E	7.07	0.07	5.67	1.33

TABLE 2

*Retentions of iron on an intake of approximately 10.4 mg a day for 4 weeks*

SUBJECT	INTAKE	EXCRETION		RETENTION
		Urine	Feces	
	<i>mg/day</i>	<i>mg/day</i>	<i>mg/day</i>	<i>mg/day</i>
A	10.36	0.06	8.50	1.80
B	10.41	0.06	8.56	1.79
C	10.45	0.07	8.01	2.37
D	9.90	0.08	7.29	2.53
E	10.45	0.07	8.61	1.77

Mean losses of iron per menstrual period were calculated for each subject from the iron lost during the two or three menstrual periods occurring during the 10 weeks; they ranged from 10.94 to 20.14 mg (table 3). The mean length of the menstrual cycle for 4 of the subjects, during the experimental period and for a later consecutive three-month period, ranged from 26 to 35 days. From the mean cycle length and

mean loss per menstrual period, the amount of iron which would have to be retained every day in order to replace the losses was calculated. For 4 subjects the values were 0.50, 0.52, 0.58 and 0.62 mg per day; for the 5th subject, whose menstrual cycles were atypical, no calculation was made.

TABLE 3

*Daily retention of iron required to replace menstrual losses*

SUBJECT	IRON LOST IN MENSTRUAL PERIODS			MEAN LOSS PER PERIOD	MEAN CYCLE LENGTH	DAILY RE- TENTION TO COVER LOSS
	I	II	III			
	mg	mg	mg	mg	days	mg
A	16.15	18.16		17.15	34	0.50
B	17.02	17.78		17.40	28	0.62
C	13.97	16.43	10.27	13.59	26	0.52
D	21.06	10.27 <sup>1</sup>	28.08	20.14	35	0.58
E	10.94			10.94 <sup>2</sup>		

<sup>1</sup> Subject D reported short periods with small loss similar to period II about twice a year. Her mean loss per period calculated on a yearly basis would, therefore, be greater than 20.14 mg.

<sup>2</sup> In the case of subject E, only one period occurred during the study. Since the intervals between periods were atypical, the daily retentions required to cover her losses were not calculated.

## DISCUSSION

Before deciding whether or not the retentions of the subjects were enough to cover their needs, possibilities of iron loss from the body other than in the menses must be taken into consideration. A complete collection of all the menstrual loss is almost impossible for every period even with the most meticulous care and with the use of the tampon-type pad. The iron content of the uncollected menses, however, falls within the experimental error. A small amount of iron is lost in hair and skin cells. In short, there are slight iron losses usually not accounted for in balance experiments, each loss small enough in itself to be within experimental error, yet when they are added together the total may be large enough to increase significantly the amount of iron which must be retained.



The adequacy of the 7-mg intake is questionable. The daily iron retentions (table 1) of subjects A, D and C were almost exactly the same as the amount needed to replace the loss in the menses (table 3); subject B retained less than was lost; and subject E retained so much that the retentions were almost sure to cover the losses although the losses were not calculated on a daily basis. If the accumulation of small losses in the uncollected menses, perspiration, hair and skin cells is taken into account, the retentions of these subjects, with the probable exception of subject E, were too near the borderline for safety.

The adequacy of the 10.4-mg intake cannot be questioned. The retentions were more than adequate to cover the menstrual and other small losses. The diet, however, was unusual in that it contained meat in every meal.

In order to assess the adequacy for women in the population as a whole of retentions of the sizes found on the two levels of intake used in this study, the most important factor to consider is the magnitude of the iron losses in the menses. After a literature survey, Frenchman and Johnston ('49) reported that the iron losses in the menses of 184 women had been investigated. The amount of iron needed every day of the year to replace the losses ranged from 0.08 to 2.6 mg (one abnormally high value was omitted). For 142 women the range was from 0.21 to 1.21 mg per day; that is, the requirement of iron to replace menstrual losses is 6 times as great for some women as for others. For 56% of the 184 women the mean daily retention of iron needed to replace the loss incurred during the few days of the menstrual period was more than  $\frac{1}{2}$  mg per day. Thus, the retention of  $\frac{1}{2}$  mg per day found on the 7-mg intake in the present study was not only too near the borderline for these subjects but it was, also, too small an amount to satisfy the needs of 56% of the 184 women studied by others. A retention high enough to cover the needs of 98 or 99% of all 184 women would be so high that extraordinary diets would have to be employed to supply it. To set that high a retention as necessary for all women would

be unwise, because most women would not need so much. A study of the frequency distribution of cases for each 0.1 mg of retention showed that beyond 1.2 mg the number of cases in each group was few. A retention of 1.2 mg, therefore, was taken as a practical amount to require for all women, although it covered the needs of only 86% of the 184 women studied. In the present study retentions on the 10.4-mg intake were large enough to more than satisfy such a requirement.

Women whose iron losses are great may absorb a higher percentage from food than other women and consequently not need a proportionately higher intake. That the "need" of the individual influences absorption to some degree under some conditions is probable, but no study has as yet been made to show whether or not large losses of iron in menses affect the percentage of absorption.

The possibility that the young women subjects of the present study might have been growing at a very slow rate and, therefore, absorbing more iron than older women must be considered. If a growth effect existed, however, it could not have been large. The possibility that these young women were absorbing more iron than men should be considered, also. Widdowson and McCance ('42) found an average absorption of 7.3% for 4 men and 12.5% for 4 women. The same authors ('48) demonstrated that female rats during the reproductive period stored more iron in their livers than male rats.

The question arises of whether or not the mean loss of approximately  $\frac{1}{2}$  mg of iron per day in the menses for the subjects of this study during the 10-week period would be similar to the mean for an extended period of a year or more. The review of the literature showed that the loss from period to period varies considerably for a given individual and that figures for at least 4, and possibly more, periods are necessary to arrive at an accurate evaluation of losses over an extended period of time. Since the mean losses for the subjects in the present study were based on only two or three menstrual periods, the values may not correspond exactly to

the mean for a year but could not be expected to vary unduly from that figure.

Several studies of iron retentions by women on intakes of 7 mg or less have been made. In some of them unusual combinations of foods were used and no retention of iron was found. On the other hand, in a study by Leverton ('41) in which the diet employed was unusual in that it contained large amounts of beef, the retentions were very large. Only one balance experiment at the 7-mg level has been carried out in recent years with women on diets customarily eaten by groups of the population. In that one (Leverton and Marsh, '42) the subjects were maintained on their usual self-chosen diets. The mean retention for 15 young women on an average intake of 7.16 mg per day was 0.41 mg, a retention similar to that found in this study.

Several investigations have been made recently with women on iron intakes in the neighborhood of 10 to 11 mg per day. Leverton and Roberts ('37), working with 4 subjects on customary diets containing 10.0, 10.7, 11.9, and 11.9<sup>2</sup> mg of iron, found retentions of 0.71, -0.20, 1.48 and 1.07, respectively. Leverton and Marsh ('42) found that 31 subjects on an intake of 8.00 to 9.99 mg retained an average of 0.96 mg, and 31 other subjects on an intake of 10.00 to 11.99 mg retained an average of 1.34 mg. Widdowson and McCance ('42) reported that 4 subjects on an average intake of 12.5 mg retained 1.8 mg. The indication is that 10 to 11 mg of iron a day may be enough to obtain a retention of 1.2 mg. Such a retention might be expected to cover the needs of women living on diets customary in this country. That 10 to 11 mg of iron a day may be more than is needed on certain unusual diets was found in the present study, in which retentions were more than adequate when beef was included in every meal. On the other hand, 10 to 11 mg may be insufficient when certain other combinations of food are used: Widdowson and McCance ('42), with 4 women

<sup>2</sup> The average values for this subject are different from those reported by the authors because a period when ferric ammonium citrate was administered has been omitted.

on a diet containing 21.1 mg (far more than 10 to 11 mg) with 40 to 50% of the calories derived from brown bread, found an average retention of only 0.2 mg per day. More studies using typical diets with an iron content of 10 to 11 mg need to be made.

In the light of the results of this study and a review of the literature, the allowance of 12 mg of iron a day recommended by the National Research Council seems to be satisfactory for women on diets which do not contain large amounts of foods that inhibit iron absorption. That a few women may need more and many do not need that much, must be recognized. Further work may demonstrate that the allowance can safely be dropped a little.

#### SUMMARY

Five women 18 years of age were maintained for 10 weeks on a controlled diet. The total period was divided into a two-week adjustment period and two 4-week experimental periods. During the first experimental period the diet contained 7 mg of iron a day and during the second period sufficient beef was added to raise the intake to 10.4 mg.

On the intake containing approximately 7 mg of iron a day, retentions for the 5 subjects were 0.26, 0.43, 0.61, 0.64 and 1.33 mg per day. The amount of iron needed per day to replace menstrual losses for 4 subjects was 0.62, 0.52, 0.58 and 0.50 mg; no value is reported for the fourth subject because the menstrual cycles were atypical. The retentions for three subjects were approximately the same as the amount needed to replace menstrual losses, and for one subject were less than the amount needed. The retentions were too low for safety because even in the three cases where retentions corresponded to menstrual losses no margin existed to care for small losses not usually included in balance experiments.

On the intake of approximately 10.4 mg of iron, retentions for the 5 subjects were 1.77, 1.79, 1.80, 2.37 and 2.53 mg per day. These retentions were more than adequate to satisfy the needs of the subjects. It should be noted that these high

retentions were obtained on a diet containing beef in every meal.

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# THE NUTRITIVE VALUE OF COD ROE AND COD LIVER

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ONE FIGURE

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During the months of February and March great shoals of cod visit the banks off the Norwegian coast for spawning, and the most extensive cod fishing of Norway takes place during this time.

The most important by-products of these fish are the cod liver and roe, which amount to several thousand tons a year each. The mean value of the catch from the years 1930-1944 was 13,000 tons of cod liver and 6,000 tons of cod roe, as reported by the Director of Norwegian Fisheries ('44).

The main part of the cod liver is used for the production of cod liver oil. A minor amount of the liver, however, has in recent years been used for the manufacture of canned pastes, often in combination with cod roe.

The roe is used in the fresh state as a valuable food by the people in coast regions. It is also canned. The main part, however, was formerly salted (cured) and exported to France and Portugal, where it was used as bait by the sardine fisheries.

The manufacture of canned cod liver-cod roe pastes has steadily increased in the last few years, and its product is now becoming of some importance for the Norwegian canning industry.

Cod liver has for a long time been the chief natural source of vitamins A and D as a supplement to common foods. Further investigations have shown that it also contains other vitamins and valuable proteins.

Investigations of cod roe reveal that it contains appreciable amounts of vitamins of the B group, proteins of high biological value, and also other substances of biological significance.

During the past few years much research work has been done in this institute to investigate the nutritive value of cod roe and cod liver. This paper summarizes previous studies and gives results of new investigations, especially concerning the biological value of the proteins.

The main constituents of the roe and liver are given in table 1, in terms of average values resulting from several analyses.

TABLE 1  
*The composition of cod roe and cod liver*

CONSTITUENTS	COD ROE	COD LIVER
	%	%
Water	71.0	27.0
Protein	23.5	7.0
Fat	1.3	65.0
Phospholipids	2.6	1.0
Ash	1.5	

## RESULTS AND DISCUSSION

### *The B vitamins of cod roe and cod liver*

*Thiamine (vitamin B<sub>1</sub>).* The thiamine content of fish and fish products has been determined by Lunde, Kringstad and Olsen ('38), biologically by the bradicardya method as well as chemically by the thiochrome method. The values vary considerably according to the condition of the roe. At the time of spawning the water content of the roe increases, and the effect seems to be that of dilution; the vitamin content calculated on the whole roe, however, is likely to remain fairly constant. The thiamine content of different samples of roe



in different seasons was found to range between 6 and 12  $\mu\text{g}$  per gram. In several samples of fresh cod liver investigated, the thiamine content ranged between 2.5 and 3.5  $\mu\text{g}$  per gram.

*Riboflavin (vitamin B<sub>2</sub>)*. Riboflavin is present in considerable amounts in cod roe and cod liver as compared with other foodstuffs. Determinations of this vitamin were carried out biologically by the growth method and by a chemical method worked out in this laboratory by Lunde, Kringstad and Olsen ('39). The latter is based upon measurement of the color, before and after reduction of the riboflavin to the leukobase, in the Pulfrich photometer using filter S45. Before a reading can be made, the extract must of course be subjected to several purification steps, including adsorption of the vitamin on Fuller's earth. The riboflavin content was determined in several samples and found to range between 6 and 15  $\mu\text{g}$  per gram of roe. In fresh cod liver the riboflavin content varied from 5  $\mu\text{g}$  to 10  $\mu\text{g}$  per gram.

*Nicotinic acid (niacin, P-P factor)*. This vitamin was determined chemically according to the method of Kringstad and Naess ('39). As compared with other sources, the cod roe and liver contain but small amounts of nicotinic acid (amide). Values between 10 and 25  $\mu\text{g}$  per gram were found.

*Pantothenic acid*. In some samples of canned roe the pantothenic acid was determined microbiologically by the method of Kringstad and Folkvord ('45), using *Streptobacterium plantarum* as the test organism. The test was carried out according to the compensation method developed by Nielsen, Hartelius and Johansen ('43) and modified by Kringstad and Folkvord ('45). This method has the advantage of taking into consideration unknown factors in the test solution which might have an influence on the growth of the test organism.

*P-aminobenzoic acid*. Para-aminobenzoic acid was determined chemically according to the method of Schmidt and Kölbl ('44). The *p*-aminobenzoic acid is diazotized and coupled with  $\alpha$ -naphthol and the resulting color measured. Tests with known amounts of *p*-aminobenzoic acid showed fairly good results, the error not exceeding 7% of the added amounts.

The concentration of pantothenic acid and *p*-aminobenzoic acid in canned roe, including that which is dissolved in the added brine, is given in table 2 (Kringstad and Folkvord, '45).

*Other vitamins of the B complex.* Although no determinations of the other members of the B complex have been carried out, it is evident from animal experiments that cod roe and liver are good sources of these vitamins. This was reported by Lunde and Kringstad ('38) and Kringstad and Lunde ('40), who performed growth experiments on rats and foxes, which were placed on a synthetic diet deprived of all B vitamins and their growth restored when small amounts of cod roe extract were added. The same growth-restoring effect was

TABLE 2  
*The pantothenic acid and p-aminobenzoic acid content of cod roe*

SAMPLE NO.	YEAR PRODUCED	D-PANTOTHENIC ACID PER 100 GM OF DRY MATTER	P-AMINOBENZOIC ACID PER 100 GM OF DRY MATTER
		mg	mg
1	1945	10.9	1.16
2	1945	12.6	1.02
3	1944	12.6	0.50
4	1940	12.6	0.47
5	1931	11.6	0.53

also obtained by these workers with the addition of defatted cod liver.

In experiments on foxes Ender and Helgebostad ('47) have recently found that on feeding stockfish a poor quality of fur resulted. By replacing some of the stockfish with cod roe, and to a minor extent with whole meal, a normal fur was developed. They ascribe this effect to a factor which protects the animals against greying (achromotrichia) and which is present in cod roe.

*Vitamin C.* Through chemical and biological analyses the presence of vitamin C in cod roe was determined by Mathiesen ('38). The values ranged between 0.2 and 0.4 mg per gram. When calculated on whole roe, the vitamin C content was found to be fairly constant, about 50 mg per gram.

*The fat soluble vitamins of cod roe and cod liver*

*Vitamin A.* The vitamin A content was investigated in some samples of fresh roe by the Carr-Price method. By saponification and isolation of the unsaponifiable matter, a greenish color developed when the  $\text{SbCl}_3$ -reagent was added to concentrated solutions in chloroform. The color seemed to be somewhat irregular as compared with that of pure vitamin A, and probably is due chiefly to other substances present. At any rate, the vitamin A content of roe must be regarded as negligible.

The mean value of vitamin A in cod liver found by spectrographical, biological and colorimetical analyses is 600 I.U. per gram.

TABLE 3

*The vitamin D content of fresh and canned cod roe and cod liver*

SAMPLE NO.	MATERIAL STUDIED	VITAMIN D PER 100 GM
		I.U.
1	Fresh roe	85
2	Fresh roe	65
3	Canned roe	80
4	Canned roe	80
5	Fresh liver	6,000
6	Canned liver	6,000

*Vitamin D.* Our determinations of vitamin D were performed on representative samples of fresh and canned cod roe and liver by the usual X-ray method. The values found are given in table 3.

*Vitamin E.* Vitamin E was determined by the authors according to the method of Emmerie and Engel ('38, '39) as modified by Kjölhede ('43). A weighed sample of the homogenized roe was saponified in an atmosphere of nitrogen and then extracted by ethyl ether. After removal of the ether in a vacuum, the unsaponifiable matter was dissolved in petrol ether and filtered through a column of Fuller's earth to remove vitamin A and other substances which might interfere with the reagent.

The values for vitamin E calculated from these measurements were astonishingly high. When calculated on the basis of dry matter, cod roe contains nearly as much of this vitamin as the amount usually found in wheat germ meal.

As these results are based only on the chemical method of determination, they have to be verified by further experiments, especially through concentration by molecular distillation.

It is interesting in this connection to note that we have not found noticeable amounts of vitamin E in cod liver, nor in any other fish products except cod roe.

TABLE 4  
*The vitamin E content of fresh and canned cod roe*

SAMPLE NO.	MATERIAL STUDIED	VITAMIN E PER 100 GM
		mg
1	Fresh roe	5.25
2	Fresh roe	7.60
3	Canned roe	5.85
4	Canned roe	7.70

### *The phospholipids of cod roe*

The relatively high content of phospholipids found in cod roe is interesting, and we have therefore investigated this fraction to determine the content of the different phospholipids.

*Extraction.* Several methods of extracting the total phospholipids from the roe were investigated. The method of Schramme ('39) was found to be the most reliable and this method was therefore used.

The roe was ground with dry  $\text{Na}_2\text{SO}_4$  and sand in a mortar and extracted with benzene-alcohol 80:20 on the water-bath in a Soxhlet apparatus for 5 hours. The solution was evaporated to dryness in a vacuum and the phospholipids dissolved in dry ether.

*Total phospholipids.* This determination was carried out according to the method of Thaler and Just ('44).

An aliquot of the ether solution of the phospholipids was placed, together with 2.5 gm MgO *p.a.*, in a platinum crucible. The ether was evaporated on a water-bath and the organic matter decomposed by combustion with the MgO at 800°C.

After cooling, the content of the crucible was dissolved in  $\text{HNO}_3\text{-H}_2\text{SO}_4$  and the phosphorus precipitated with ammonium-molybdate as usual. From the found phosphorus the phospholipids are calculated by the conversion factor 25.45, corresponding to a stearo-oleo-lecithin with molecular weight of 788 and containing 3.929% phosphorus.

*Lecithin + sphingomyelin.* The method used for this determination was that of Thannhauser, Benotti and Reinstein ('39). The choline, which is liberated by hydrolysis of the

TABLE 5  
*The phospholipids of cod roe*

PHOSPHOLIPID	SAMPLE 1	SAMPLE 2
	%	%
Phospholipid content of roe	2.66	2.70
Sphingomyelin content of total phospholipids	42.6	45.2
Lecithin content of total phospholipids	45.6	45.0
Cephalin content of total phospholipids	11.8	9.8

phospholipids with methyl alcoholic HCl, is precipitated by Reinecke salt. The precipitate is washed with chloroform, alcohol and ether and then dissolved in acetone. The resulting color is measured in the Zeiss Stepphotometer using the filter S53. From a calibration curve with known amounts of choline-reineckate the content of lecithin + sphingomyelin is found by multiplying by the factor 1.92.

*Sphingomyelin.* This determination was carried out according to the method of Thannhauser and Setz ('36), by precipitating the sphingomyelin as reineckate. The weight of sphingomyelin-reineckate multiplied by 0.788 gives the weight of sphingomyelin.

*Cephalin* [= total phospholipids - (lecithin + sphingomyelin)]. Table 5 gives the results of the phospholipid determinations of two samples of cod roe.

*The digestibility and biological value of cod roe  
and cod liver proteins*

The main constituent of cod roe is protein, which, on an average, amounts to 23.5% of the fresh roe, or 81% as calculated on the basis of dry matter. The eggs of the roe serve as a nutrient source for the developing organism, and it is therefore to be expected that the proteins have a high biological value and probably contain all essential amino acids. Although fat is the chief component of the cod liver and the protein only amounts to 7%, it is of importance to ascertain whether this protein is of any biological significance.

The method used in these investigations was essentially that of Mitchell ('24), with slight modifications. It has been shown by Boas Fixsen ('30) that vitamins of the B group are of importance in determining the biological value of proteins by animal experiments. Through lack of B factors, especially B<sub>1</sub>, loss of appetite causes a decrease in food consumption, which results in a low caloric value for the food consumed. Hence the biological value of the proteins will be found to be too low.

In our experiments adult rats, with a weight of 170 to 230 gm for males and 160 to 185 gm for females, were used. The animals were placed in separate cages provided with a screen bottom, arranged on a funnel in such a way that feces and urine could be collected separately.

The composition of the basal nitrogen-free diet used is given in table 6. In addition, the animals received 20 µg thiamine, 20 µg riboflavin, 20 µg pyridoxine, 20 µg *p*-aminobenzoic acid, 100 µg nicotinic acid, and 300 µg Ca-pantothenate. These B factors were given orally (in water solution) from a calibrated pipette daily.

The nitrogen-free diet was fed for an 8-day period and feces and urine were collected for the last 5 days. In order to prevent losses of volatile nitrogen compounds during storage, due to decomposition, the feces were placed in concentrated sulphuric acid. To the urine-collecting flask a few

milliliters of 50% acetic acid were added, and the funnel and bottom screen of the cages were washed with diluted acetic acid. The urine was stored under toluene in a refrigerator.

During a following period of 8 days a diet containing 8% of the protein to be tested was fed. The food was given *ad libitum* and the food consumed recorded each day. Feces and urine were collected as described above.

The total feces and urine excreted by each rat during the 5-day period were digested according to Kjeldahl, and finally diluted with water to a desired volume. Aliquots of these solutions were used for nitrogen determinations according to the semimicro-Kjeldahl method.

TABLE 6  
*Composition of nitrogen-free diet used*

INGREDIENT	AMOUNT
	%
Potato starch	78.2
Sucrose	6.0
Arachis oil	8.0
Cod liver oil	2.0
Salt mixture (McCollum)	5.0
Calcium carbonate	0.8

The nitrogen content of the basal diet was negligible, and the nitrogen excreted during feeding on this diet is therefore assumed to be endogenous nitrogen.

Experiments were carried out on fresh, canned and dried cod roe, and on samples of defatted fresh and canned cod liver. For comparison, tests were also carried out on commercial casein.

The digestibility of the proteins was calculated according to the following formula:

$$D = 100 \cdot \frac{N \text{ intake} - (N \text{ in feces} - N \text{ in feces endogenous})}{N \text{ intake}}$$

The biological value was calculated according to Mitchell's formula, namely:

$$BV = 100 \cdot \frac{N \text{ absorbed} - (N \text{ in urine} - N \text{ in urine endogenous})}{N \text{ absorbed}}$$

N absorbed was calculated according to the expression:  

$$\text{N absorbed} = \text{N intake} - (\text{N in feces} - \text{N in feces endogenous}).$$

It is obvious that the excretion of endogenous nitrogen is subject to variations which may influence the results to a noticeable degree. In order to get as reliable values as possible, both fecal and urinary endogenous nitrogen were determined for each animal before and after each test period.

In table 7 the digestibility and biological values of different samples of cod roe and of defatted fresh and canned cod liver are given. The results of a test on a commercial dried casein are also included.

TABLE 7  
*Digestibility and biological value of cod roe and cod liver proteins*  
*(The values are given as means of 10 rats.)*

	FRESH ROE	CANNED ROE	DRIED ROE	FRESH LIVER	CANNED LIVER	CASEIN
Digestibility	81.3	80.8	79.2	68.7	70.5	84.4
Standard deviation of the means	1.26	1.35	0.78	1.19	1.39	1.22
Biological value	88.4	87.6	88.2	76.4	77.7	78.8
Standard deviation of the means	0.71	0.92	0.40	0.90	0.70	1.26

*Growth experiments on rats with cod roe  
as protein source*

In confirmation of the results obtained in the metabolic experiments, and especially to discover whether the proteins contain all essential amino acids, growth experiments with cod roe as the sole protein source were performed.

The composition of the basal diet was the same as that described above, except that the potato starch was replaced by purified rice starch. The ingredients were mixed with the test material to make a protein content of 14% as calculated on dry matter. No synthetic B vitamins were added in these experiments, since the roe has been shown to be a good source of growth factors of the B complex.



Rats with a weight of 50 to 60 gm, 4 males and 4 females, were used in each test. The diet was given ad libitum and the food consumed determined weekly. The experiment lasted for 7 weeks. In table 8 the values are given as means for two rats (placed in each cage) per week. As will be seen, the average weight increase for the animals on fresh cod roe was 118 gm for males and 102 gm for females. The corresponding values for rats on canned roe were 86 and 84, respectively. The rats in the last group did not take the food as well as those on the fresh roe, and the food consumption

TABLE 8  
*Growth experiment with fresh and canned cod roe*  
(Duration 7 weeks)

DIET	NUMBER OF RATS	SEX	TOTAL FOOD INTAKE	TOTAL PROTEIN INTAKE	TOTAL WEIGHT INCREASE	PROTEIN EFFICIENCY RATIO
			gm	gm	gm	
Fresh	4	♀	1,351	189	407	2.15
cod roe	4	♂	1,562	218	471	2.16
Canned	4	♀	1,171	164	336	2.05
cod roe	4	♂	1,210	169	344	2.03

of these animals was somewhat lower. The lesser weight increase of the animals on canned roe can therefore not be taken as an indication of the lack of some nutritive factor. When the weight increase is calculated per gram of protein consumed, the values for canned roe are only slightly lower than those for fresh, being 2.04 and 2.16, respectively.

Finally, an experiment with canned cod roe as the only nutrient source was carried out, 4 male rats with a weight of about 60 gm being fed canned cod roe for three weeks. The animals took the food well and showed excellent growth response during the whole period. The growth curves are shown in figure 1.

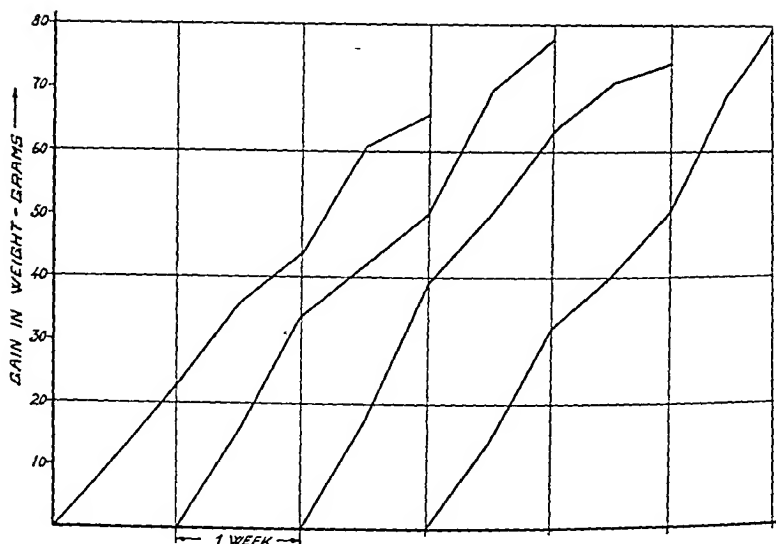


Figure 1

## SUMMARY

In earlier studies it was found by extensive investigations that cod roe and cod liver are valuable sources of vitamins of the B complex. Cod roe especially was found to be rich in B vitamins, and to contain also appreciable amounts of vitamin C. The high content of vitamin A and D in cod liver is well known and has been appreciated for years. In the present paper new data from further investigations of the nutritive value of cod roe and cod liver are given.

Only traces — if any — of vitamin A were found (Carr-Price reaction) in cod roe. The vitamin D content amounted to about 80 I.U. on the average per 100 gm. The colorimetric determination, made according to the method of Emmerie and Engel ('38, '39), gave evidence of the presence of considerable amounts of vitamin E. Calculated on dry matter, the vitamin E content in roe is nearly as high as in wheat germ meal.

The phospholipid content of cod roe was found to be 2.70%, and the phospholipids shown to consist of 45% lecithin, 44% sphingomyelin and 11% cephalin.

Investigations of the nutritive value of cod roe proteins were carried out, and gave results of 81% digestibility and 88% biological value. No significant differences among fresh, canned and dried roe were found. For comparison, the same experiments were performed with casein. The results showed that the biological value of cod roe proteins is considerably higher than that of casein. The proteins of defatted fresh and canned cod liver were also investigated in the same way, and gave values of 69% for digestibility and 77% for biological value. No noticeable difference was found between fresh and canned cod liver with respect to digestibility and biological value of the proteins.

Growth experiments on rats fed cod roe proteins indicated the presence in the proteins of all essential amino acids.

#### CONCLUSIONS

Cod roe as well as cod liver has been shown to be a good source for substances of biological significance. Cod roe is valued chiefly owing to its high content of B vitamins and first-grade proteins. Cod liver is valued mainly because of its high content of fat, the fat soluble vitamins A and D, and B vitamins.

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# CONCENTRATIONS OF VARIOUS CONSTITUENTS IN BLOOD OF DAIRY COWS DURING STAGES OF TERMINAL GESTATION AND INITIAL LACTATION

## I. EFFECT OF PREPARTAL DIET ON SERUM TOCOPHEROLS <sup>1</sup>

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The importance of tocopherols (vitamin E) in animal nutrition suggested a study of this nutriment in the parturient dairy cow. Although considerable information on the function of this vitamin in laboratory animals is available, similar reports on dairy cattle are few. Recent studies have indicated that deficiencies of tocopherols may result in cardiac disorders (Gullickson and Calverley, '46). According to Harris et al. ('47), dietary supplementation of a typical herd ration with tocopherols during lactation improved the general health of cows and increased the fat content of milk and yield of "4% milk," but these observations have not been substantiated (Fountaine and Parrish, '48; Gullickson et al., '48; Phillips et al., '48; Whiting et al., '49). Supplementation of

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rations of dairy cows during the terminal stages of gestation augmented the secretion of tocopherols in colostrum and early milk (Parrish et al., '47).

The investigation reported herein was designed primarily to gain additional information on the role of tocopherols in dairy cattle by determinations of changes in the concentration of this vitamin in the blood serum during stages of terminal gestation and initial lactation of animals fed typical barn rations, unsupplemented or supplemented.

#### PROCEDURES

##### *Grouping, feeding and management of experimental animals*

Pertinent facts concerning the grouping of the experimental heifers and cows and the duration and levels of vitamin supplementation for each group are presented in table 1. Vitamin A supplementation during the two weeks preceding the scheduled date of parturition was doubled, since investigations of Sutton et al. ('45) revealed a marked tendency for concentrations of this vitamin in blood to decrease during this stage of pregnancy. When tocopherols were administered with vitamin A, the ratio of the two vitamins was uniform throughout the trial. All tocopherols used were in the free, unesterified form. Further details of the feeding and management of the experimental animals have been reported by Parrish et al. ('47).

##### *Collection and preservation of blood samples*

Samples of venous blood for analysis were obtained from the various groups of cows at the prepartal stages indicated in table 2 and from all groups immediately after parturition (0-6 hours) and also one, two, three, 7, 14, 27 and 28 days later. Collections were made from 14 to 16 hours following daily administration of vitamin supplements. After the blood had clotted, serum was obtained by centrifugation. When immediate analysis was not possible, the serum was stored in

tightly closed bottles protected from light and refrigerated at 4° C.

### *Analytical procedures*

The analytical method used was the Quaife and Biehler ('45) modification of the Quaife and Harris ('44) procedure, except as follows: Removal of Skelly-solve B to obtain the lipid residue containing tocopherols was accomplished by

TABLE 1

*Schedule of daily supplementation of diets of pregnant heifers and cows*  
(The  $\alpha$ - $\gamma$  tocopherols were either 50 or 90%  $\gamma$  form)

GROUP	SUB-GROUP	NUMBER OF ANIMALS	DAYS PREVIOUS TO DATE OF EXPECTED PARTURITION	
			28 — 14	13 — 0 <sup>1</sup>
I	a	5	No supplement	No supplement
	b	8	500,000 I.U. vit. A alcohol	1,000,000 I.U. vit. A alcohol
	c	6	500,000 I.U. vit. A ester	1,000,000 I.U. vit. A ester
II	—	6	500,000 I.U. vit. A alcohol and 0.5 gm $\alpha$ - $\gamma$ tocopherol	1,000,000 I.U. vit. A alcohol and 1 gm $\alpha$ - $\gamma$ tocopherol
III	a	3	10 gm mixed tocopherol	10 gm mixed tocopherol
	b	1	0.5 gm $\alpha$ - $\gamma$ tocopherol	1 gm $\alpha$ - $\gamma$ tocopherol
	c	1	4 gm mixed tocopherol	4 gm mixed tocopherol
	d	1	5 gm $\alpha$ - $\gamma$ tocopherol	5 gm $\alpha$ - $\gamma$ tocopherol
VI <sup>2</sup>	—	1	No supplement	No supplement

<sup>1</sup> Vitamin supplementation discontinued at parturition.

<sup>2</sup> Cow milked through gestation, without a dry period.

evaporation under suction, using a water bath at 50° to 60°C. for heating. The hydrogenated extract was warmed in a water bath at 35°C. prior to the addition of the analytical reagents. A Coleman spectrophotometer with a special cell holder that accommodated 7"  $\times$  7/8" matched absorption tubes was used in the assay. Photometric readings were made at a wavelength setting of 520 m $\mu$ . The instrument was calibrated using pure natural  $\alpha$ -tocopherol.<sup>4</sup>

<sup>4</sup> Supplied by M. L. Quaife, Distillation Products, Inc., Rochester, N. Y.

*Method of adjusting analytical data*

Attempts were made to obtain prepartal blood samples at intervals that would represent not only definite periods of vitamin supplementation (table 1) but also specific stages in the terminal stages of gestation (table 2), both of which affect tocopherol concentration in the serum. Since parturition frequently did not occur on the anticipated date, in several instances the first 4 prepartal samples collected according to the outlined schedule of supplementation did not correspond

TABLE 2

*Prepartal stages at which blood samples were scheduled to be collected from experimental cows, compared with times of actual collection*

SCHEDULED COLLECTION	ACTUAL COLLECTION (Averages and ranges)			
	Group I	Group II	Group III	Group IV
35 <sup>1</sup>	38 (51-25)	44 (58-35)	42 (49-35)	..
28 <sup>2</sup>	31 (41-18)	32 (50-21)	34 (37-28)	28
14 <sup>3</sup>	16 (27-9)	19 (38-8)	18 (26-14)	14
7	8 (11-6)	8 (14-5)	8 (11-7)	7
3	3	3	3	3
1	1	1	1	1

<sup>1</sup> Start of trials, a pre-supplementation period.

<sup>2</sup> Immediately prior to start of supplementation of cows that received vitamins.

<sup>3</sup> Immediately prior to increase of supplementation of cows that had levels of vitamin intake changed.

to the expected gestational stage. When this occurred, adjustments were made in the data from individual cows so that averages could be computed on a period basis. Interpolations from graphs representing the data from the cows were employed in those cases in which the time of blood collection did not correspond to the scheduled periods. The data, actual or interpolated when necessary, were averaged by dietary groups (tables 3 and 4).

When the data were examined with respect to breed and number of lactation periods, no consistent differences attributable to either of these two factors were found; hence they were disregarded in the computation of means.



## RESULTS

*Trends of tocopherol levels in blood serum of cows that received no tocopherol supplements*

Cows directly off pasture frequently had high levels of blood serum tocopherols. Typical of these high values, expressed as  $\mu\text{g}$  per 100 ml, are 1,473, 898, 873, 737 and 724. The levels of serum tocopherols for other cows in corresponding stages of pregnancy that received only barn rations for the preceding two to 8 weeks were never more than 450  $\mu\text{g}$ .

As parturition approached, a downward trend was exhibited in the tocopherol concentrations in blood serum of cows receiving no supplemental tocopherols (group I, table 3), the rate of decrease generally becoming more pronounced during the days immediately before parturition. The minimum values usually occurred on the second or third day *post partum*, after which the concentrations gradually increased (group I, table 4). At the end of the first month of lactation, the average levels were higher than at the initial period one month before parturition.

Comparisons of data from groups I-a, -b and -c indicate that supplementation of the prepartal diet with vitamin A had no significant effect upon either the magnitude of tocopherol concentrations or their trends in the blood serum.

The cow of group IV received a diet similar to that of the cows in group I-a (table 1) but was milked throughout gestation without the usual dry period. The levels of tocopherols in her blood serum, in contrast to those of other cows, increased slightly as gestation approached termination (table 3). The maximum concentration was observed on the first day *post partum* and the minimum on the 7th day (table 4).

*Trends of tocopherol levels in blood serum of cows that received tocopherol supplements*

Tocopherol levels of blood serum of preparturient cows that received vitamin A alcohol and 0.5 to 1 gm of tocopherol supplements daily (group II, table 3) were more than 15% higher

TABLE 3  
Prepartal changes in tocopherol concentrations of blood serum from dairy cows subjected to various dietary treatments

GROUP	SUB-GROUP	DAILY SUPPLEMENT	STAGES (DAYS PREPARTAL) OF BLOOD COLLECTION						
			Pre-suppl. <sup>1</sup>		Initial suppl.		Terminal supplementation		
			35 <sup>2</sup>	28	14	7	3	1	
I	a	None	....	433 (3) <sup>3</sup>	340 (5)	347 (5)	328 (5)	290 (5)	
			....	351-557	302-405	325-373	306-352	266-353	
	b	Vitamin A alcohol	588 (5)	508 (6)	402 (6)	365 (7)	298 (8)	286 (8)	
			371-898	396-755	266-588	286-427	204-384	180-371	
	c	Vitamin A ester	384 (3)	530 (5)	395 (6)	320 (6)	268 (6)	248 (6)	
II			266-470	279-873	266-526	237-483	167-483	176-450	
		Group mean	511 (8)	499 (14)	381 (17)	345 (18)	296 (19)	275 (19)	
		Vitamin A alcohol and 0.5-1 gm tocopherol	690 (5)	535 (6)	466 (6)	414 (6)	391 (6)	388 (6)	
			310-1,473	390-792	359-532	301-518	328-510	307-545	
III	a	10 gm mixed tocopherol	351 (3)	479 (3)	1,268 (3)	1,333 (3)	1,201 (3)	1,164 (3)	
			198-427	297-724	890-1,764	930-1,836	965-1,594	919-1,622	
	b <sup>4</sup>	0.5-1 gm tocopherol	384	396	569	559	557	378	
	c <sup>4</sup>	4 gm tocopherol	724	588	1,187	1,278	1,271	1,136	
	d <sup>4</sup>	5 gm tocopherol	619	378	919	861	805	813	
IV <sup>4,5</sup>		None	...	283	341	353	353	365	

<sup>1</sup> See table 1.

<sup>2</sup> See table 2.

<sup>3</sup> Number of samples represented in the means enclosed in parentheses.

<sup>4</sup> Only one cow in the classification.

<sup>5</sup> Milked through entire gestation.

TABLE 4  
*Postpartal changes in the tocopherol concentrations of blood serum from dairy cows receiving various prepartal dietary treatments*

GROUP	SUB-GROUP	DAILY PREPARTAL SUPPLEMENT <sup>1</sup>	STAGES (DAYS) POST PARTUM										
			0 <sup>2</sup>	1	2	3	7	14	21	28			
I	a	None	276 (5) <sup>a</sup> 254-316	260 (5) 210-297	240 (5) 192-285	<i>Tocopherols, μg/100 ml</i> 261 (5) 198-310					410 (5) 298-613	420 (5) 322-463	575 (5) 490-631
	b	Vitamin A alcohol	271 (8) 155-371	248 (8) 136-340	227 (8) 142-291	236 (8) 99-365	329 (8) 204-508	454 (2) 314-593	528 (2) 424-632	..	..	..	
	c	Vitamin A ester	234 (6) 115-433	233 (6) 149-384	213 (6) 155-340	228 (6) 155-340	275 (6) 192-470	..	..	..	..	..	
		Group mean	260 (19)	244 (19)	226 (19)	240 (19)	306 (19)	423 (7)	451 (7)	575 (5)	575 (5)	575 (5)	
	II		Vitamin A alcohol and 0.5-1 gm tocopherol	350 (6) 285-508	292 (6) 229-390	255 (6) 192-297	279 (6) 210-340	323 (6) 266-458	..	..	..	..	
			Av. Range	350 (6) 285-508	292 (6) 229-390	255 (6) 192-297	279 (6) 210-340	323 (6) 266-458	..	..	..	..	
III	a	10 gm mixed tocopherol	1,011 (3) 848-1,281	1,044 (3) 848-1,269	1,007 (3) 904-1,151	852 (3) 563-1,003	593 (3) 393-899	582 (3) 378-885	643 (3) 452-900	659 (3) 435-896	659 (3) 435-896		
	b <sup>4</sup>	0.5-1 gm	396	279	347	285	369	501	513	616	616		
	c <sup>4</sup>	4 gm tocopherol	1,003	891	848	823	836	799	799	799	842		
	d <sup>4</sup>	5 gm tocopherol	817	650	582	557	396	594	680	799	799		
IV <sup>4,5</sup>		None	347	396	359	316	241	379	..	..	..		

<sup>1</sup> All supplementation discontinued at parturition.

<sup>2</sup> First sample after parturition.

<sup>3</sup> Number of samples represented in the means enclosed in parentheses.

<sup>4</sup> Only one cow in each classification.

<sup>5</sup> Milked throughout gestation.

after 14 days of supplementation than those of animals that did not receive additional tocopherols (group I). The high values in groups I-b and II at the initial periods possibly were due to residual effects of tocopherols obtained from pasture (Cabell and Ellis, '42), from which several cows were removed immediately before collection of first blood samples. The general prepartal trends of the tocopherol levels of groups I and II were similar, but the accelerated rate of decrease for group II began later, at the time of parturition. Concentrations of tocopherols in blood serum from group II immediately after parturition (table 4) remained higher than those of the controls (group I), but after 7 days of lactation the levels were approximately the same.

The addition of from 4 to 10 gm of tocopherols to the rations of animals in group III (table 3) increased markedly the levels of this vitamin in the blood serum. At high levels of intake, maximum levels of serum tocopherols generally were reached one to two weeks before parturition. Even though the rate of increase of tocopherols in the blood serum of the cow of group III-d was greater than that of the cow of group III-c, the latter consistently maintained a higher concentration throughout the trial. When 0.5 gm of tocopherol was introduced into the ration of the cow of group III-b, the concentration of this vitamin increased in the blood serum, but a similar amount of tocopherols given to cows in group II did not prevent the downward trend. Failure of supplementation to increase tocopherol content in serum from cows of group II probably was due to higher pre-supplementation levels.

At stages of supplementation 13 days to one day before parturition, concentrations of tocopherols in the blood serum of cows receiving 10 gm of this vitamin daily (group III-a) were approximately 4 times higher than those of the controls (group I). However, even this high level of supplementation did not prevent the downward trend of tocopherol concentrations as parturition approached.

The residual effects of prepartal tocopherol supplementation on postpartal concentrations of this vitamin in the blood serum seemed to be related to the levels of intake. When daily additions to the diet were 0.5 to 1 gm, and 10 gm, the minima occurred, respectively, from one to two days and from 7 to 14 days postpartally. After the termination of the decline in the control cows (group I), the differences between the tocopherol levels in the serum of these animals and of those receiving tocopherol supplements before parturition gradually diminished. Four weeks after calving, blood serum tocopherol levels of cows of group III-a were only about 15% higher than those of group I, and were within the same range as those of cows in groups III-b, -c and -d.

The various levels of vitamin supplementation produced no noticeable advantage in either the thriftiness or the reproductive health of the cow; there was no apparent effect on the incidence of retention of placental membranes.

#### DISCUSSION

Declines in concentrations of tocopherols in the blood serum of dairy cattle during the stages of terminal gestation and early lactation apparently are characteristic for cows subjected to conventional feeding and management practices. The trends are similar to those observed for vitamin A and carotene (Sutton et al., '45; Wise et al., '47). Diversity in the reported values of tocopherols in the blood of pregnant women suggests that the changes in human subjects do not follow so definite a pattern as has been noted for cattle (Straumfjord and Quaife, '46; Varangot, '42; Varangot et al., '43). It is improbable, however, that the parturient decreases in the concentrations of vitamin E in the blood of cattle are unique for this species.

The specific factors involved in the reduction of tocopherol levels have not been determined. Deficiency of the vitamin obviously was not an etiological factor at the levels of intake investigated, inasmuch as the decreases occurred regardless of dietary supplementation. Withdrawal of tocopherols from

the blood in the fortification of the early mammary secretions (Parrish et al., '47) possibly played a role in the reduction, but similar declines in the serum of a mammetomized cow receiving normal rations (Fountaine and Parrish, '48) indicated that other metabolic changes associated with parturition and initial lactation probably were involved to a greater extent than mammary functions *per se*.

Present information on factors affecting concentrations of tocopherols in blood is inadequate to explain the causes of some of the observed differences. In contrast to responses observed in other experimental subjects, the upward prepartal trend in the serum of the cow of group IV introduces the question of whether omission of the normal dry period affected tocopherol concentrations in the blood. Further investigation is needed to ascertain if the reaction was an individual peculiarity or is a typical response of cows that continue milk production throughout gestation.

The prepartal level of tocopherol intake was one of the primary factors affecting concentrations of this vitamin in the blood of the experimental subjects. The dietary responses were in accord with those noted in human beings (Couperus, '43; Quaife and Harris, '44; Wechsler et al., '41, '43), rabbits (Minot, '44), calves (Latschar, '47) and lactating cows (Fountaine and Parrish, '48; Harris et al., '47; Whiting et al., '49). A report by Kaay ('47) revealed further that the tocopherol contents of blood from cows, pregnant and non-pregnant, varied markedly according to the feed supplied. His results, in harmony with values observed in the first samples of blood serum from cows used in the current study, indicated that pasture grazing greatly increases the tocopherol levels. According to calculations of vitamin E intake, cows fed a typical barn ration consume approximately 1 gm daily (Phillips et al., '48), whereas cows eating 100 lb. of grass daily ingest 7.1 gm (Cabell and Ellis, '42). Therefore, the levels of tocopherols in the blood of well-fed cows would not be expected to change to any marked degree when small amounts of tocopherols are added to the ration.

Tocopherol supplementation during the terminal weeks of gestation affected levels and trends of this vitamin in the blood of cows not only prepartally but also postpartally. These residual effects indicated that the additional tocopherol intake augmented the body reserves.

The time of collecting the blood samples in relation to the ingestion of supplemental tocopherols may have been another factor related to the concentrations of this vitamin detected in blood serum. Couperus ('43) and Quaife and Harris ('44) reported that maximum concentrations of tocopherols in human blood were attained 6 hours after oral administration of a single dose. The time required for the levels to return to normal seemed to vary with the amount ingested. It is probable that the rate of absorption in ruminants is slower and more uniform than in monogastric animals. Though in the present study blood samples were drawn at a specific time, 14 to 16 hours after feeding the tocopherols, the stage represented in the absorption cycle was not ascertained.

In harmony with the report of Kaay ('47), the well-being of heifers and cows and their reproductive performance were unrelated to levels of tocopherol in the diet and in the blood. Thus barn rations seemingly provided sufficient amounts of this vitamin to meet minimum needs for normal completion of gestation and initiation of lactation. Since the present investigation covered only a small segment of the gestation-lactation cycle, longer periods of supplementation might have yielded different results; but the findings of Gullickson et al. ('44) using vitamin E-low rations indicate that this is improbable. Nevertheless, a more comprehensive exploration is warranted.

#### SUMMARY

1. Determinations were made of tocopherol concentrations in the blood serum of dairy cows receiving typical barn rations, unsupplemented or supplemented, during stages of terminal gestation and initial lactation.
2. Tocopherol concentrations in the blood serum of dairy cows restricted to typical barn rations decreased slowly dur-

ing the terminal month of pregnancy. The decline generally became more pronounced within a few days before parturition and reached a minimum level the second day *post partum*, after which a gradual but continuous rise ensued.

3. Addition of vitamin A supplements to the diet of the preparturient dairy cow had no significant effect on tocopherol levels in the blood serum.

4. Addition of tocopherols (0.5 to 1 gm) to the diet resulted in concentrations of blood serum tocopherols averaging more than 15% higher than those in the controls.

5. Addition of large tocopherol supplements daily to typical barn rations of dairy cows during the concluding month of gestation resulted in an increase in tocopherol levels in blood serum, the highest level of tocopherol supplementation (10 gm daily) eliciting the greatest response (approximately 4 times higher than in the controls).

6. The higher levels of tocopherol supplementation, 4 gm to 10 gm daily, counteracted the gradual downward prepartal trend in blood serum concentrations of this vitamin which was characteristic of serum from cows receiving no tocopherol supplements, but did not prevent the rapid decline in the immediate parturient period.

7. After cessation of tocopherol supplementation at parturition, the *post partum* trends of blood serum tocopherols were similar for all groups; however, high levels of prepartal supplementation delayed the time of attainment of the minimum level and tended to maintain higher concentrations subsequently.

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# NET PROTEIN AND GROWTH-PROMOTING VALUES OF THREE DIFFERENT TYPES OF YEAST PREPARED UNDER IDENTICAL CONDITIONS

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## ONE FIGURE

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A previous investigation (Goyco and Asenjo, '46) has indicated that though the nitrogen content and true digestibility of different samples of yeasts were quite similar, there was marked difference between the biological values of strains of *Torula* and brewers' yeasts and, consequently, between their respective net protein values. Two different strains of *Torula utilis* exhibited biological values which were over 21% below those observed in a sample of brewers' yeast studied at the same time. This difference in biological values could have been due to the influence of the growing mash or to the processing used in their elaboration, since they were prepared with different nutrient mediums and at different places.

The purpose of the following two experiments was to study the protein quality of different species and varieties of yeasts which had been prepared under identical conditions.<sup>1</sup>

<sup>1</sup>Pure cultures of *Torula utilis* No. 1084, *Saccharomyces cerevisiae* ("Gebruder Meyer") No. 52 (Brewers' Yeast No. 52), and Fleischmann's Stock Bakers' Yeast No. 51 (Bakers' Yeast No. 51) were furnished by us to Mr. Carlos Vincenty, of the Puerto Rico Industrial Development Company, who prepared a quantity of each one of these yeasts by identical techniques. The growing mash used in their elaboration was Puerto Rican blackstrap molasses clarified and diluted to 5° Brix, with three grams of ammonium sulfate and 2 gm of ammonium acid phosphate (secondary) added per liter of the diluted molasses.

The writers are most grateful to Mr. Vincenty for his valuable cooperation.

## EXPERIMENTAL

*Determination of digestibility, biological, and net protein values*

In order to ascertain the digestibility, biological, and net protein values of the proteins of the different yeasts, the technique recommended by Mitchell and Carman ('24) was used with some modifications. As the details of this procedure have been already described in a previous communication (Goyco and Asenjo, '46), they will not be repeated here.

The tests lasted for 10 days and were divided into two periods: a three-day preliminary period when no samples were collected, and a 7-day experimental period during which urine and feces samples were collected daily. All diets used in the metabolism experiments contained about 8% protein on a wet basis ( $N \times 6.25$ ). Their composition is given in table 1.

TABLE 1  
*Composition of diets*

CONSTITUENTS <sup>1</sup>	STANDARD- IZING DIET	TORULA UTILIS NO 1084	BREWERS' YEAST NO 52	BAKERS' YEAST NO 51	TORULA UTILIS NO 1084 AND 0.5% DL- METHIONINE
	%	%	%	%	%
Dried, ether extracted whole egg	6.50				
Dried yeast		14.45	16.80	16.95	14.45
Modified Osborne and Mendel salts	3.00	3.00	3.00	3.00	3.00
Sucrose	10.00	10.00	10.00	10.00	10.00
Sodium chloride	1.00	1.00	1.00	1.00	1.00
Vegetable oil (Mazola)	9.00	9.00	9.00	9.00	9.00
Cod liver oil	1.00	1.00	1.00	1.00	1.00
Corn starch	66.50	58.55	56.20	56.05	58.05
Cellu-flour	3.00	3.00	3.00	3.00	3.00
DL Methionine					0.50
Protein content	4.44	7.62	8.06	8.12	7.66

<sup>1</sup> One drop daily of B complex concentrate was fed to each animal: 200  $\mu$ g of thiamine; 20  $\mu$ g of riboflavin; 2.25  $\mu$ g of pyridoxine; 6.2  $\mu$ g of pantothenic acid; 500  $\mu$ g of niacin-niacinamide, and 0.4 units of filtrate factor.

The results of the initial and final standardizing periods are shown in table 2. These two values, when plotted, permitted the interpolation, during the testing period, of a specific value for each animal under observation.

From the nitrogen metabolism data assembled in table 3 the coefficients of true digestibility and biological and net protein values were computed in the usual way. The values thus obtained are reported in table 4.

TABLE 2

*Daily excretion of metabolic and endogenous nitrogen during initial and final standardizing periods*

	BODY WEIGHT		FOOD INTAKE	FECAL NITROGEN	METABOLIC NITROGEN IN FECES PER GRAM OF FOOD	URINARY NITROGEN	ENDOGENOUS NITROGEN IN URINE PER 100 GM WEIGHT
	Initial	Final					
	gm	gm	gm	mg	mg	mg	mg
<i>Initial period: dried egg protein ration containing 0.71% N</i>							
Average of 10 rats	55.4	67.1	6.5	7.7	1.2	12.7	20.8
Standard deviation	3.4	4.6	0.7	0.9	0.1	1.2	0.9
<i>Final period: dried egg protein ration containing 0.71% N</i>							
Average of 10 rats	103.3	110.0	7.7	10.2	1.3	14.9	14.0
Standard deviation	11.9	11.1	0.7	1.2	0.1	1.7	0.7

Although its digestibility was slightly higher, *Torula utilis* exhibited biological and net protein values much lower than either one of the *S. cerevisiae* strains studied. However, when 0.5% DL-methionine was added to the *Torula utilis* ration the coefficient of digestibility as well as the biological and net protein values increased to the very high figures of 90.2, 88.3, and 44.1%, respectively.

In the case of the two strains of *S. cerevisiae*, Brewers' No. 52 and Bakers' No. 51, the coefficient of digestibility and the biological and net protein values were almost identical in each case, 79.9 and 80.7, 58.4 and 58.9, and 22.2 and 22.7, re-

TABLE 3  
*Nitrogen metabolism of rats on yeast rations*  
 (Results expressed on daily basis)

	BODY WEIGHT		FOOD INTAKE	NITROGEN			
	Initial	Final		Intake	In feces	In urine	Balance
	gm	gm	gm	mg	mg	mg	mg
<i>Torula utilis</i> No. 1084 ration							
Average of 10 rats	60.1	58.1	2.49	30.4	7.6	29.0	- 6.2
Standard deviation	4.5	4.2	0.6	7.3	1.8	3.6	3.7
<i>Brewers' yeast</i> No. 52 ration							
Average of 10 rats	60.1	66.0	4.70	60.6	17.9	31.6	11.1
Standard deviation	4.4	6.0	0.8	10.6	3.2	3.7	7.3
<i>Bakers' yeast</i> No. 51 ration							
Average of 10 rats	68.7	74.3	4.62	60.1	17.4	31.8	10.9
Standard deviation	7.1	9.7	1.0	13.6	3.8	4.9	6.1
<i>Torula utilis</i> No. 1084 and 0.5% DL-methionine ration							
Average of 10 rats	81.4	95.8	6.28	76.6	15.5	21.6	39.5
Standard deviation	11.1	12.3	1.0	12.4	2.2	2.6	8.0

spectively. These values were lower than those previously reported by us (Goyco and Asenjo, '46) for a different strain of *S. cerevisiae*.<sup>2</sup>

### *Growth-promoting studies*

For the growth studies, 28-day-old male Wistar albino rats, weighing 54 to 57 gm, were transferred from the stock colony to round double-bottom cages, three to a cage.

<sup>2</sup> Fleischmann Type 2019. No definite information could be obtained on the composition of the mash used to grow this yeast; probably it was a cereal mash.

Experimental yeast diets were fed to them ad libitum, the composition of these diets being the same as of those used in the previous experiment except for the percentage of yeast, which was increased to about 18% protein (table 5), and the percentage of cornstarch, which was reduced proportionally. A diet containing purified casein<sup>3</sup> as the sole source of protein was used for comparison. All animals were supplemented

TABLE 4

Coefficient of true digestibility, biological value and net protein value of the nitrogenous substances present in yeast

	TORULA UTILIS NO. 1084	BREWERS' YEAST NO. 52	BAKERS' YEAST NO. 51	TORULA UTILIS NO. 1084 AND 0.5% DL-METHIONINE
(a) % Protein ( $N \times 6.25$ ) in the yeast samples	55.37	47.68	47.25	55.37
(b) Total fecal nitrogen <sup>1</sup>	7.6	17.9	17.4	15.5
(c) "Metabolic nitrogen" in feces <sup>1</sup>	3.0	5.7	5.8	8.0
(d) Food nitrogen in feces (b-c) <sup>1</sup>	4.6	12.2	11.6	7.5
(e) Total nitrogen intake <sup>1</sup>	30.4	60.7	60.1	76.7
(f) Nitrogen absorbed (e-d) <sup>1</sup>	25.8	48.5	48.5	69.2
(g) Total urinary nitrogen <sup>1</sup>	29.0	31.6	31.8	21.7
(h) Endogenous nitrogen in urine <sup>1</sup>	11.4	11.4	11.9	13.6
(i) Food nitrogen in urine — (g-h) <sup>1</sup>	17.6	20.2	19.9	8.1
(j) Food nitrogen retained (f-i) <sup>1</sup>	8.2	28.3	28.6	61.1
(k) Biological value: $\frac{i}{f} \times 100$	31.8	58.4	58.9	88.3
(l) True digestibility coefficient: $\frac{f}{e} \times 100$	84.8	79.9	80.7	90.2
(m) Net protein value ( $a \times k \times l$ )	14.9	22.2	22.7	44.1

<sup>1</sup> Results expressed in milligrams of nitrogen per day. Values are average of 10 rats.

with the vitamins listed in table 1. All rations were kept in the refrigerator until needed. The trials lasted for 4 weeks.

The growth curves of the different groups of rats appear in figure 1. Examination of them shows that the ration containing *Torula utilis* as the sole source of protein gave the poorest growth performance, yet this same ration when sup-

<sup>3</sup> Vitamin test casein, General Biochemicals, Inc., Chagrin Falls, Ohio.

TABLE 5

*Growth-promoting value of proteins in rations*  
(Results expressed on 4-week basis)

RATION	PROTEIN IN RATION BY ANALYSIS	NO. OF RATS PER GROUP	AVERAGE INITIAL WEIGHT	AVERAGE GAIN IN WEIGHT	AVERAGE DIET CONSUMED	AVERAGE PROTEIN CONSUMED	GROWTH- PROMOTING VALUE OF PROTEIN a/b
				"a"		"b"	
	%		gm	gm	gm	gm	
Torula yeast							
No. 1084	17.62	9	56.7	22.4	138.6	24.94	0.9
Bakers' yeast							
No. 51	17.69	9	54.8	43.3	176.2	31.71	1.4
Brewers' yeast							
No. 52	17.94	9	55.1	57.4	187.7	33.78	1.7
Purified casein	18.03	12	54.6	60.6	175.0	31.50	1.9
Torula yeast No. 1084 plus 0.5% DL- methionine	18.06	6	56.1	84.3	233.1	41.95	2.0

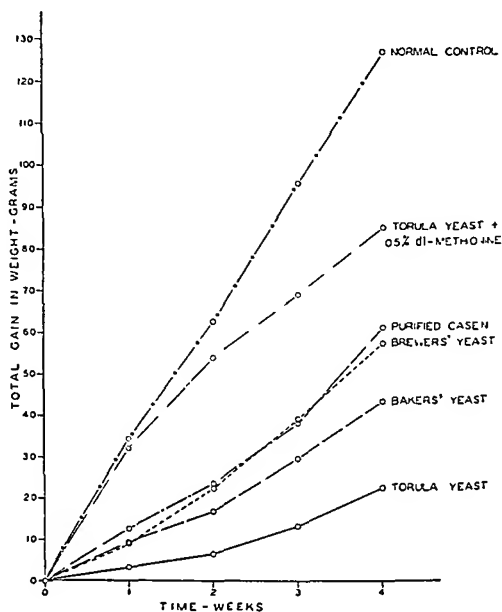


Fig. 1 Growth curves of groups of rats fed the different rations.



plemented with 0.5% DL-methionine gave the best performance of all.

Rations containing the two different strains of *S. cerevisiae* supported growth at somewhat different rates. The brewers' strain paralleled the growth performance of the ration containing casein, while bakers' strain had a poorer rate of growth though still much better than that of the *Torula* ration. Table 5 presents a summary of the details of this experiment, with calculations of the average gain in weight per gram of protein consumed.

The gain in weight of normal rats on our stock colony ration No. 1 (Goettsch, '46) for a similar 4-week period averaged 125 gm, a much superior gain to that obtained on any of the rations fed in this experiment.

#### DISCUSSION

The true digestibilities thus obtained were as follows: 84.8 for *Torula* yeast No. 1084; 90.2 for *Torula* yeast No. 1084 supplemented with methionine; 79.9 for Brewers' Yeast No. 52; and 80.7 for Bakers' Yeast No. 51.

Growth-promoting, biological, and net protein values of *Torula* yeast protein were decidedly lower than those demonstrated by the two strains of *S. cerevisiae*. As these three yeasts were prepared under identical conditions, it would seem that the lower nutritional value exhibited by *Torula utilis* under the conditions of this experiment was due to an inherent property of these pseudo-yeast proteins, and not to the environmental and nutritional factors that acted on the organism during its growth and development.

In a previous experiment (Goyco and Asenjo, '46) with a sample of *Torula utilis* No. 1084 prepared in England in a molasses-salts mash, the biological value reported was higher than the one obtained here — 48.8 as compared to 31.8 for the sample prepared in Puerto Rico in a similar, although not identical, mash. Even the former value of 48.8 was much lower than those biological values recorded by the present

authors in 1946 and by others (Sure and House, '49; Hughes and Hauge, '45), for different strains of *S. cerevisiae*.

In the present experiment the food intake was somewhat lower than that observed in the previously reported trial with samples of *Torula* manufactured in England (Goyco and Asenjo, '46). As a result of this lower food intake a negative nitrogen balance was induced in the animals, as can be seen in table 3. However, the rats consumed over 150 Cal. in non-protein constituents of the diet and not less than 500 mg of nitrogen per kilogram of body weight per day (compare Boas Fixsen, '30).

The great improvement noted in the performance of *Torula* protein on addition of 0.5% DL-methionine to the ration was remarkable. The recorded biological and growth-promoting values were almost three times as high as the ones for *Torula* protein alone, and are of an order exhibited only by the very best quality proteins. It is apparent that *Torula* yeast has an appreciable amount of its methionine in an unavailable form.<sup>4</sup>

These findings agree with those of Klose and Fevold ('45), who observed that the nutritional inadequacy of yeast protein could be corrected by the addition of methionine. The small amount of methionine added to the diet under discussion apparently stimulated the food intake of the experimental animals. There was an average intake of 2.5 gm of diet per day, without methionine; the addition of 0.5% methionine increased the intake to 6.3 gm per day.

Although the two strains of *S. cerevisiae*, Bakers' No. 51 and Brewers' No. 52, exhibited almost identical biological values (58.9 and 58.4, respectively), they differed significantly in growth-promoting values. Strain 51 had a growth-promoting value of 1.4, while 52 had a much higher one, 1.7. This difference between the two strains would indicate that the protein in the brewers' strain is more suitable for stimulating

<sup>4</sup>The three yeasts used were analyzed for methionine by the method of McCarthy and Sullivan ('41) using pepsin as the hydrolyzing agent. As fed they contained respectively: yeast No. 1084—0.67%; yeast No. 51—0.91%, and yeast No. 52—0.69% methionine.

growth than that in bakers' yeast. Both, however, seem to function equally well for maintenance purposes.

### SUMMARY

The nutritive value of the proteins of three different types of yeasts prepared under identical conditions was evaluated by means of the nitrogen balance and growth-promoting methods.

The *Saccharomyces cerevisiae*, Bakers' No. 51 and Brewers' No. 52, and *Torula utilis* No. 1084 were found, respectively, to be 80.7, 79.9, and 84.8% digestible; to possess biological values of 58.9, 58.4, and 31.8; to have net protein values of 22.7, 22.2, and 14.9; and to possess a protein efficiency of 1.4, 1.7, and 0.9.

With the exception of the coefficient of digestibility, the nutritive coefficients of *S. cerevisiae* strains were generally much higher than those of the *Torula*.

The protein of *Torula utilis* No. 1084, when supplemented by 0.5% methionine, gave the following nutritive coefficients: digestibility, 90.2; biological value, 88.3; net protein value, 44.1; and protein efficiency, 2.0.

The two strains of *S. cerevisiae*, Bakers' No. 51 and Brewers' No. 52, had similar nutritive coefficients with the exception of that for protein efficiency, which was higher in the case of strain 52.

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# THE ESSENTIAL AMINO ACID CONTENT OF SEVERAL VEGETABLES <sup>1</sup>

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The general use of microbiological methods for the determination of amino acids has produced a considerable volume of published data on the amino acid contents of pure proteins, meat, eggs, other animal products, and cereal grains. However, very little data on the amino acid contents of vegetables are available. Stokes et al. ('45) give values for the essential amino acid content of carrots, peas, and potatoes. Whole dried corn has been investigated for its amino acid content by a number of workers (Horn et al., '46, '48; Schweigert, '47; Jones et al., '48) but very little if any data are available on the amino acid content of fresh sweet corn.

In this paper are reported the percentages of essential amino acids in broccoli, cauliflower, and several varieties of sweet corn and carrots. The consumption of these vegetables, especially carrots, has increased in the U. S. in the last few years (Clark et al., '47).

## EXPERIMENTAL

Samples of each vegetable, or variety of vegetable, were collected at random, on the same day, from carefully controlled irrigated plots at the University of Arizona Vegetable Research Farm. Thus the different varieties of vegetable received the same fertilizer and irrigation treatment and

<sup>1</sup> Aided in part by a grant from the Williams-Waterman Fund for the combat of dietary diseases.

were subject to the same climatic variations. Two samples of broccoli of the Italian sprouting variety were assayed; one was collected at a different date than the other. Two varieties of cauliflower, Snowball and Snowdrift; 4 varieties of sweet corn, Yellow Hybrid, Kingcross, Tendergold and Seneca Chief; and 9 varieties of carrots, Imperator, Rubicon, Touchon, Tempe, Hutchinson, Morse's Bunching, Danver's Half Long, Red Core Chatenay and Scarlet Nantes were assayed. The vegetables were steam blanched for 5 min. and then dried overnight in a Stewart Warner dehydrator at 150°F. Moisture determinations were made on the dehydrated materials.

For assay of the essential amino acids other than tryptophan, accurately weighed quantities of the dried vegetables were refluxed 24 hours with 20% hydrochloric acid. The hydrolysates were evaporated on a hot plate to a syrupy consistency, the pH adjusted to 6.8–7.0, and the hydrolysates then diluted to proper volume and filtered.

For the assay of tryptophan, accurately weighed samples were autoclaved at 121°C. for 5 hours with 5 N sodium hydroxide (Krehl et al., '46). Fifty milligrams of cysteine were added at the beginning of the hydrolysis and again after one to two hours. As was found by Kuiken et al. ('47), this technique prevented the destruction of tryptophan. The final results were multiplied by two to correct for the racemization of tryptophan caused by the alkaline hydrolysis.

The hydrolysates were assayed for the essential amino acids almost exactly as indicated by Henderson and Snell ('48). Wasserman tubes (13 mm x 100 mm) were used instead of the 18 mm x 150 mm tubes specified by the above authors. For growing bacteria 10% filtered tomato juice was added to the media. For tryptophan both *Lactobacillus arabinosus* 17-5 and *Streptococcus faecalis* R were used.

For all the amino acids<sup>2</sup> except isoleucine, values obtained are expressed on the basis of 100% activity for the L-isomer and 50% activity for the DL-isomer. For DL-isoleucine the

<sup>2</sup>All amino acids used for the standard curves were manufactured by Merck and Company.

values obtained are expressed on the basis of 39% of the activity of the L-isomer (Brickson et al., '48). Where both the L-form and the DL-form of the amino acid were available commercially, the responses of the assay organism to each were determined.

To check on the hydrolysis and microbiological procedure, recoveries were run. Amounts of amino acids approximately equal to those naturally present in the samples were added before hydrolysis. Also one sample of each vegetable was refluxed 40 hours with 20% HCl and the amino acid content

TABLE 1  
*Recovery of amino acids added before hydrolysis*

AMINO ACID	BROCCOLI	CAULIFLOWER	CARROT	SWEET CORN
	%	%	%	%
L-Arginine	91	94	100	103
L-Histidine	96	95	95	100
DL-Isoleucine	102	100	97	108
L-Leucine	96	104	107	107
L-Lysine	110	95	92	..
DL-Methionine	103	109	97	96
DL-Phenylalanine	105	97	97	114
DL-Threonine	101	113	100	90
DL-Tryptophan	95	100	105	102
DL-Valine	108	113	93	113
Mean	101	102	98	104

of this hydrolysate was compared with that of the 24-hour hydrolysate to determine whether the latter period was sufficient to release each one of the amino acids completely.

## RESULTS

The percentages of recovery of the amino acids added previous to hydrolysis are given in table 1. For all amino acids the recoveries are as good as can be expected using microbiological methods. The mean of the recoveries for sweet corn (104%) is slightly higher than for the other vegetables. It is possible that sweet corn contains substances, or

produces substances during hydrolysis, that slightly increase the response of microorganisms to several of the essential amino acids, especially to phenylalanine, leucine, and isoleucine.

Forty hours of hydrolysis did not liberate appreciably more of any amino acid than did 24 hours of hydrolysis, but did give 10% lower results for phenylalanine.

The response of *L. arabinosus* to DL-leucine, DL-phenylalanine, and DL-tryptophan, and of *L. mesenteroides* to DL-histidine and DL-lysine, was one-half as much as the response to the respective L-isomers. However, L-leucine, L-histidine and L-lysine were used in the standard curves.

In table 2 are given the percentages of essential amino acids in the vegetables, calculated both on the dry basis and on the basis of protein content ( $N \times 6.25$ ). Referring to the work of Dunn ('47), and assuming that the amino acids are present in whole egg in optimum proportions, it follows that all these vegetable proteins are deficient in lysine and isoleucine. Cauliflower proteins compare fairly well with beef muscle proteins, being definitely deficient in three amino acids— isoleucine, leucine, and lysine—and possibly in two others, phenylalanine and arginine. According to Dunn ('47), beef muscle protein is not optimum in 5 of the essential amino acids. Broccoli is deficient in 8, being optimum only in arginine and methionine. Carrot protein is deficient in all the essential amino acids. Sweet corn is approximately optimum in histidine, leucine, phenylalanine and valine. A mixture of broccoli, cauliflower and sweet corn would afford proteins balanced in all the essential amino acids except lysine and isoleucine.

The variation in the percentages of amino acid in the proteins of the different varieties of corn and carrots is striking. Even though soil and climatic conditions were exactly the same for the different varieties tested there is no conclusive proof that one variety will be consistently better in any one amino acid than any other variety. It is well recognized that tests for nutritive value of different varieties of food crops



TABLE 2  
Essential amino acid content of some vegetables

NO. VARIETIES TESTED	BROCCOLI		CAULIFLOWER		CARROTS		SWEET CORN		WHOLE EGG (DUNN, '47)
	1	2	2	2	9	9	4	4	
PROTEIN CONTENT	29.4 (25.3-33.5)	24.3 (22.6-25.9)	24.3 (22.6-25.9)	24.3 (22.6-25.9)	6-0 ± 0.24 <sup>1</sup> (5.3 ± 7.0)	6-0 ± 0.24 <sup>1</sup> (5.3 ± 7.0)	16.1 ± 0.98 (14.3 ± 18.3)	16.1 ± 0.98 (14.3 ± 18.3)	
AMINO ACID %	Sample	Prot.	Sample	Prot.	Sample	Prot.	Sample	Prot.	Prot.
Methionine	0.70 (0.64-0.76)	2.4 (2.3-2.5)	0.63 (0.63-0.63)	2.6 (2.4-2.8)	0.057 ± 0.003 (0.039-0.074)	0.96 ± 0.07 (0.76-1.25)	0.26 ± 0.007 (0.24-0.27)	1.7 ± 0.11 (1.4-1.9)	3.1
Phenylalanine	0.97 (0.92-1.02)	3.3 (3.0-3.6)	0.88 (0.84-0.92)	3.7 (3.7-3.6)	0.25 ± 0.015 (0.18-0.34)	4.1 ± 0.24 (3.4-5.1)	0.98 ± 0.16 (0.73-1.41)	6.0 ± 0.59 (5.0-7.5)	5.4
Valine	1.14 (1.10-1.17)	3.9 (3.3-4.6)	1.54 (1.44-1.64)	6.4 (5.6-7.3)	0.34 ± 0.014 (0.27-0.42)	5.7 ± 0.24 (4.7-6.8)	1.07 ± 0.079 (0.89-1.26)	6.6 ± 0.11 (6.3-7.2)	7.1
Leucine	1.80 (1.60-1.99)	6.1 (5.9-6.3)	1.76 (1.66-1.85)	7.2 (7.1-7.4)	0.33 ± 0.018 (0.28-0.43)	5.6 ± 0.072 (4.6-6.4)	1.74 ± 0.091 (1.51-1.93)	10.8 ± 0.27 (10.2-11.3)	9.2
Isoleucine	1.05 (0.85-1.24)	3.6 (3.4-3.7)	1.05 (0.93-1.16)	4.4 (4.1-4.6)	0.26 ± 0.012 (0.23-0.29)	4.4 ± 0.14 (3.9-4.8)	0.61 ± 0.110 (0.51-0.68)	3.8 ± 0.33 (3.4-4.4)	6.7
Threonine	1.10 (0.99-1.21)	3.8 (3.6-3.9)	1.20 (1.16-1.23)	5.0 (4.8-5.1)	0.24 ± 0.005 (0.21-0.26)	4.0 ± 0.12 (3.4-4.5)	0.67 ± 0.071 (0.54-0.87)	4.1 ± 0.17 (3.9-4.6)	5.3
Lysine	1.54 (1.45-1.63)	5.3 (4.9-5.7)	1.35 (1.30-1.39)	5.6 (5.4-5.8)	0.18 ± 0.012 (0.13-0.22)	3.1 ± 0.19 (2.3-4.0)	0.61 ± 0.070 (0.56-0.71)	3.8 ± 0.07 (3.6-3.9)	7.3
Histidine	0.54	1.6	0.50 (0.44-0.57)	2.1 (2.0-2.2)	0.09 ± 0.000 (0.08-0.11)	1.5 ± 0.08 (1.2-1.9)	0.41 ± 0.020 (0.38-0.48)	2.6 ± 0.05 (2.5-2.7)	2.4
Arginine	1.71 (1.47-1.94)	5.8 (5.8-5.8)	1.18 (1.13-1.22)	4.9 (4.7-5.0)	0.21 ± 0.020 (0.10-0.30)	3.6 ± 0.39 (1.4-5.5)	0.79 ± 0.080 (0.69-0.99)	4.9 ± 0.48 (4.0-6.2)	5.7
Tryptophan	0.36 (0.32-0.39)	1.3 (1.2-1.3)	0.37 (0.34-0.40)	1.6 (1.3-1.8)	0.036 ± 0.0005 (0.029-0.043)	0.6 ± 0.07 (0.53-0.78)	0.081 ± 0.004 (0.075-0.090)	0.5 ± 0.00 (0.5-0.5)	1.6

<sup>1</sup> Standard deviation of the mean.

must be conducted over a number of years before any differences can be assumed as significant. However, our results indicate that the amino acid constituents of plant proteins may be affected by certain factors. This also is borne out by the work of Schweigert ('47), who reported considerable differences in the leucine content of the protein in two different samples of milo. The protein content of this author's samples varied much less than the protein content of either our corn or carrot samples.

#### SUMMARY

The content of essential amino acids was determined in broccoli, two varieties of cauliflower, 9 varieties of carrots, and 4 varieties of sweet corn. The protein of cauliflower as compared with whole egg protein is fairly well balanced in essential amino acids. It is definitely optimum in methionine, valine, threonine, histidine, tryptophan, and possibly arginine and phenylalanine. Sweet corn protein is approximately optimum in histidine, leucine, phenylalanine and valine, and broccoli protein in arginine and methionine. Carrot protein is deficient in all the essential amino acids.

The percentage of amino acids in the above plant proteins varied in the different samples. The variation is not attributed to difference in variety.

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# A PARADOXICAL RELATIONSHIP BETWEEN SERUM LEVEL AND LIVER CONTENT OF VITAMIN A<sup>1</sup>

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ONE FIGURE

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At the present time at least two views exist regarding the relationship between blood levels and the amount of vitamin A stored in the liver. Steigmann and Popper ('44) believe that if a sufficiently large number of cases is studied, some degree of parallelism may be observed between blood and liver levels. This has been attributed to the free vitamin A concentration in the liver (Glover, Goodwin and Morton, '47). Lewis, Bodansky, Falk and McGuire ('42), on the other hand, feel that blood levels of vitamin A are regulated by some means other than the amount stored in the liver. On reviewing the evidence in support of these two views it seemed probable that conditions such as age, sex, amount of supplementation and the degree of vitamin A depletion might determine which process regulates the relationship between vitamin A blood and liver levels.

The work of Lewis et al. ('42) was done on young male and female rats, three to 4 weeks old, that had been given supplements prior to depletion tests. It was therefore decided to examine this blood-liver relationship in older rats that had not received vitamin A supplements, and in similar rats under the influence of varying degrees of vitamin A depletion.

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## METHODS AND PROCEDURES

Rats used were from a commercially supplied Wistar strain. The females weighed 125 to 150 gm and the males 175 to 225 gm at the beginning of the study.

Blood samples were taken by cardiac puncture and total livers removed from 51 female and 61 male rats; 27 males and 19 females were on normal diets<sup>2</sup> and the remainder were on a vitamin A-free diet<sup>3</sup> for periods ranging from 4 to 20 weeks.

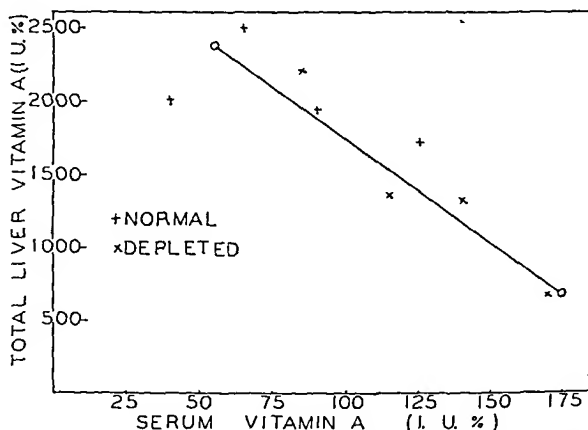


Fig. 1 The relationship between serum vitamin A and total liver content in normal and partially depleted female rats.

All animals were fasted 12 hours prior to taking the samples. Serum vitamin A was determined on duplicate samples by the method of Bessey et al. ('46). The livers were weighed, finely sectioned, thoroughly mixed and aliquots taken for the determination of vitamin A by the method of Oser et al. ('43).

## RESULTS

A summary of the results is presented in figure 1 and tables 1 and 2. The points for the graph were determined by arranging the data into groups for which the interval for serum levels of vitamin A was 25 I.U./100 ml. The average serum

<sup>2</sup>Rats on a Purina Laboratory Chow diet.

<sup>3</sup>Rats on a vitamin A test diet, U.S.P. XII.

level in each group was plotted against the corresponding average value for the total vitamin A in the liver. In this figure the points were determined and plotted separately for normal and depleted females. The line of regression drawn between the points for normal and depleted female rats was determined by the method of least squares. A relation similar to the above for females was noted for normal male rats.

TABLE 1

*Average weight, serum level and total liver content of vitamin A in normal and partially depleted rats*

DIET	NO. OF RATS	WEIGHT		SERUM LEVEL			TOTAL LIVER LEVEL		
		Ave.	S.D.	Ave.	S.D.	Range	Ave.	S.D.	Range
<i>Females</i>									
		<i>gm</i>		<i>I.U. %</i>			<i>I.U.</i>		
Normal	27	140	± 15	70	± 37	( 55-125)	2,226	± 705	(1,019-3,280)
Vit. A-free, 4 wks.	6	160	± 18	135	± 24	(110-175)	1,161	± 172	( 850-1,360)
Vit. A-free, 8 wks.	6	180	± 12	115	± 26	( 55-150)	1,368	± 681	( 715-1,960)
Vit. A-free, 16 wks.	6	190	± 11	115	± 34	( 80-165)	1,436	± 946	( 141-3,020)
Vit. A-free, 20 wks.	6	190	± 10	140	± 26	(125-175)	1,073	± 511	( 600-2,130)
<i>Males</i>									
Normal	19	200	± 24	125	± 49	( 55-175)	3,021	± 1727	(1,517-6,230)
Vit. A-free, 4 wks.	10	250	± 25	135	± 46	( 25-200)	362	± 156	( 100-600)
Vit. A-free, 8 wks.	20	279	± 26	95	± 52	( 20-165)	174	± 126	( 39-499)
Vit. A-free, 12 wks.	12	304	± 23	65	± 45	( 0-155)	61	± 26	( 27-106)

The relationship between the serum vitamin A level and liver content of the partially depleted male rats is presented in table 2. These rats were divided into two groups on the basis of their liver content of vitamin A and not according to the time on the vitamin A-deficient diet. In group I were placed those rats whose livers contained only apparent vitamin A; the remaining depleted male rats, whose total liver vitamin A content ranged between 103 and 499 I.U., were placed in group 2.

TABLE 2

*The serum level and liver content of two groups of partially depleted male rats*

	NO. RATS	TOTAL LIVER CONTENT		SERUM LEVEL	
		Ave.	Range	Ave.	S.D.
Group I	19	63 <sup>1</sup>	I.U. 36-95	75	I.U. 48
Group II	23	277	103-499	120	44
Serum level difference (group II — group I)				45 <sup>2</sup>	

<sup>1</sup> This value should be labelled "apparent vitamin A," since the color developed by adding the Carr-Price reagent to the extracts from these livers did not produce a characteristic color for vitamin A. A similar observation has been described by Mattson et al. ('47).

<sup>2</sup> The standard error of the difference between the mean serum levels as determined by the formula  $\sigma_D = \sqrt{\frac{\sigma_1^2}{N_1} + \frac{\sigma_2^2}{N_2}}$  (Arkin and Colton, '46) was found to be 14 I.U., thus indicating the statistical significance of the difference between the mean serum levels for groups I and II.

It is evident from an examination of these data that an inverse relationship exists between the blood and liver levels of vitamin A in normal male and female rats within the ranges tested. It may also be noted that this inverse relationship holds true in the case of the vitamin A-depleted female animals. The coefficient of correlation<sup>4</sup> for the line of regression as drawn in figure 1 was found to be -0.95, indicating that the relationship is significant.

In the male rats the depletion was more severe than in the females, as is indicated by the lower vitamin A content of the

$$^4 r = \sqrt{1 - \frac{sy^2}{y^2}} \text{ (Arkin and Colton, '46).}$$



liver (see tables 1 and 2). Here a direct relationship was found between the blood and liver levels; that is, as the liver content decreased the serum level also decreased.

From table 1 it will be noted that the average serum vitamin A levels were lower and the liver content higher in the normal female group of rats than in those subjected to the stress of depletion. In the males, however, the serum level and liver content were both lower in the depleted group than in the normal group.

It may be noted that there was a sharp decline in the average amount of vitamin A in the livers of rats after they were on a deficient diet for 4 weeks. The males were more severely affected than the females. Some female rats had as high as 3,000 I.U./total liver after being on a vitamin A-free diet for 20 weeks, whereas in the male group (12 rats), after a similar diet for 12 weeks, all except one had a liver content of vitamin A of less than 100 I.U. The inverse relationship between blood and liver levels held for the former but not for the latter case.

The degree of stress placed on these animals was not sufficient to produce any of the usually accepted physical signs of vitamin A deficiency. The male rats gained weight throughout the test period, whereas the females, after 12 weeks of depletion, did not show an increase in weight but remained constant up to 20 weeks.

#### DISCUSSION

The evidence presented in figure 1 supports the hypothesis that there is a regulatory mechanism controlling the blood vitamin A level of rats which, under the conditions of this experiment, operates to give an inverse relationship between the blood level and liver content of vitamin A. This regulatory process was found to function in both normal male and female rats and also in those females subjected to vitamin A depletion. It is obvious, however, that the range of this relationship is limited and that it would not apply under conditions of excessive supplementation or extreme depletion. This

latter limitation is shown by the group of male rats on the vitamin A-free diet, in which it will be noted that a low liver content is associated with a low serum level. This change in relationship may be attributed to a breakdown of the regulatory mechanism by the depleted state of the animal. It is of course possible that certain sexual factors may also affect this process.

Sex differences in the rate of loss of liver stores have been previously summarized by Brenner et al. ('42) as favoring the females. This view is further substantiated by the present findings; namely, that in general the male rats were more severely depleted of their vitamin A liver stores in a period of 4 weeks than were the females over a 20-week period.

If low liver content and serum levels of vitamin A are an indication of a deficiency state, it is difficult to account for the continued increase in weight of all male rats, especially in those whose serum levels were zero and whose livers contained less than 100 I.U. of apparent vitamin A. It seems to the author that either vitamin A is not as important a factor in the growth and development of older rats as it is for younger rats, or that a measure of the liver content and serum level is not adequate for determining a deficiency state.

This study confirms the rapid loss of the vitamin A liver depots in the first 4 weeks as noted by Popper and Brenner ('42) and Davies and Moore ('35). The evidence contained in histological studies by Popper and Brenner ('42) and the more recent work of Glover and Morton ('48) suggests that this early and great loss may involve a specific labile type of vitamin A, stored in the hepatic cells as a protein complex, which in turn is readily available for liberation into the blood stream. The more gradually depleted form may represent that vitamin A stored in the Kupffer cells.

It is impossible, with the evidence at hand, to present a satisfactory explanation for the two relationships found to exist between liver content and serum level of vitamin A. It is suggested on the basis of the inverse relationship that perhaps tissue demand is a more potent factor governing serum

blood levels than is liver depot content. Various factors such as age, sex, disease and amount of vitamin A stored undoubtedly affect this relationship.

In conclusion it may be stated that the evidence gained from this study plus the observations described by other workers (see above) support the view that it is difficult to assess the body's need or its reserve of vitamin A on the basis of blood level per se. In light of these findings it seems evident that additional investigation must be made in order to determine whether these factors affect the serum vitamin A level in man.

#### SUMMARY

An examination was made of the relationship between serum level and liver content of vitamin A in normal rats and those under the influence of varying degrees of vitamin A depletion. The following points were observed:

(1) An inverse relationship existed between blood and liver levels of vitamin A in normal male and female rats and those depleted female animals whose liver content ranged as low as 600 I.U./total liver.

(2) When the total liver content fell below 600 I.U. in the males there was a parallelism between blood and liver levels.

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## ERRATA

The Journal of Nutrition, vol. 39, no. 2, October, 1949.

HEBER R. BALDWIN AND CLARENCE P. BERG

The influence of optical isomerism and acetylation upon the availability of tryptophan for maintenance in man.

*Page 204, line 28, the word "available" should have been "unavailable." Corrected line reads —*

vert the acetyl-D-tryptophan into an unavailable product; one

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BENJAMIN H. ERSHOFF

Protective effects of soybean meal for the immature hyperthyroid rat.

*The word "reduction" appearing in line 4, page 279 under Summary should have been "elevation." The corrected line reads —*

the attendant elevation in basal metabolic rate.



# ASCORBIC ACID METABOLISM OF OLDER ADOLESCENTS <sup>1</sup>

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ONE FIGURE

(Received for publication April 18, 1949)

## INTRODUCTION

A review of the literature in the field of ascorbic acid requirements showed few data on adolescent subjects. The recommended allowances for ascorbic acid for adults, children 8 to 12 years of age, pre-school children and infants were derived from experimental data on those age groups (National Research Council, '45). The allowances for children over 12 years of age apparently were not based on experimental studies on this age group but were derived from data obtained from studies on adults and younger children.

So far as the present authors know, the first controlled study on the vitamin C requirements of adolescents was re-

<sup>1</sup>Published as Technical Paper No. 578 with the approval of the Director of the Oregon Agricultural Experiment Station. Contribution of the Nutrition Laboratory of the Home Economics Experiment Station in cooperation with the School of Home Economics, Oregon State College, and the Bureau of Human Nutrition and Home Economics, U. S. Department of Agriculture.

<sup>2</sup>The data in this paper are taken from the thesis presented by Bessie L. Davey to the Graduate School faculty of Oregon State College in partial fulfillment of the requirements for the Ph.D. degree, June, 1949. Further details will be found in the thesis.

ported from this laboratory (Storvick et al., '47). Eight young adolescents, 4 boys and 4 girls who were 12 through 14 years of age, participated in that study, in which fasting plasma ascorbic acid determinations were made daily during three one-week periods when the ascorbic acid intake was controlled. For these subjects it was found that the daily allowance of ascorbic acid recommended by the National Research Council ('45) resulted in plasma ascorbic acid values lower than those which they attained during the saturation period but well above 0.6 mg%, the quantity considered adequate by Butler ('40).

In order to extend the knowledge of ascorbic acid requirements to the older adolescent age group, studies were conducted in which 16 Oregon State College freshmen, 16 through 19 years of age, participated as subjects.

The various levels of ascorbic acid intake for this study were selected in order to determine: (1) the concentrations of ascorbic acid in the plasma of subjects receiving the National Research Council recommended allowance for ascorbic acid, and (2) whether or not a small decrease in ascorbic acid intake, i.e., 10 mg less than the recommended allowance, had a significant effect on the concentration of ascorbic acid in the plasma.

#### EXPERIMENTAL

The work reported in this paper was done during the academic years 1946 to 1948. The studies during the first year were divided into 4 periods of 7 days each in order to compare the daily values of plasma ascorbic acid when the subjects received the following: first period, unrestricted diets; second period (or saturation period), a daily supplement of 200 mg of crystalline ascorbic acid<sup>3</sup> in addition to the ascorbic acid in the food; third period, the total daily ascorbic acid intake recommended by the National Research Council; and 4th period, a total intake of 10 mg less than they received during the third period. During the second year the studies were

<sup>3</sup> Acknowledgment is made to Merck and Company Inc., Rahway, New Jersey, for a generous supply of crystalline ascorbic acid.

divided into three periods of 10 days each, allowing more time to adjust to each level of ascorbic acid intake. The daily ascorbic acid intakes for the three periods were the same as those for the second, third and 4th periods, respectively, of the preceding year's studies.

TABLE 1  
*Description of subjects*

YEAR	SUBJECT	AGE	MEAN WEIGHT		HEIGHT	
			lb.		in.	
1946-47	Girls	MG	18	119	61½	
		WH	16	125	67	
		BR	19	130	65½	
		PS	18	111	59½	
	Boys	JC	18	155	71½	
		VD	18	156	69½	
		JJ	18	157	71	
		GS	18	170	71½	
1947-48	Girls	NA	18	117	65½	
		BD	18	116	65	
		MF	18	145	65½	
		RR	18	153	64½	
	Boys	TC	18	186	73½	
		DE	18	182	73½	
		WP	18	129	66	
		DR	18	151	70½	

The ages, weights, and heights of the human subjects are given in table 1. These individuals were fed in a college dormitory. All of the foods which they ate were weighed and recorded. The general plan for controlling the intake of ascorbic acid from food was the same as that described in the previous paper by Storvick et al. ('47). Samples of foods which might be presumed to contain ascorbic acid were analyzed<sup>4</sup> by the method of Loeffler and Ponting ('42). Through-

<sup>4</sup>Details of the experimental methods and procedures are available in Technical Bulletin 12 (1947) published by the Oregon Agricultural Experiment Station and from the thesis submitted to the Graduate Faculty of Oregon State College by Bessie L. Davey ('49).

out the experimental studies blood samples were taken daily before breakfast and the reduced ascorbic acid in plasma was determined by the micro method of Farmer and Abt ('36).

## RESULTS

Table 2 presents a summary of the means for plasma ascorbic acid concentrations for each subject. During the 1946-47 studies the plasma values for all of the days in the first, or orientation, period of each study were included in the calculations, since no attempt was made to control the ascorbic acid intake for this period. For the second, third and 4th periods the plasma values were averaged for the last 5 days, thus allowing two days for the subjects to adjust to the new levels of intake. In the 1947-48 studies the mean values were calculated both on the basis of the last 8 days and the last 5 days, allowing two and 5 days, respectively, for adjustment to new levels. By comparison of these two sets of calculations we hoped to ascertain whether the longer experimental periods were really necessary. All of the daily ascorbic acid intake values were averaged in every experimental period.

The significance of the differences between the means was tested statistically. The difference is considered significant if it is twice as large as the standard deviation of the differences.

### *Girls*

In the first, or orientation, period of the girls' study in 1946-47, three of the 4 girls showed near-saturation values according to the results of fasting plasma ascorbic acid determinations (fig. 1A). The mean fasting plasma ascorbic acid values were 1.00 mg% or higher for three of the subjects during this period.

A rise in plasma ascorbic acid values was observed in all cases with the ingestion of saturation levels of ascorbic acid during the second period. Although three subjects appeared to be "saturated," WH may possibly not have been, since on

TABLE 2  
Summary of mean plasma ascorbic acid concentrations and deviations from the mean for each subject during experimental periods on different levels of intake

SUBJECT	UNRESTRICTED		"SATURATION"				NIO RECOMMENDED ALLOWANCE				10 MG IS THAN NIO RECOMMENDED ALLOWANCE			
	All days	mg %	Last 8 days	mg %	Last 5 days	mg %	Last 8 days	mg %	Last 5 days	mg %	Last 8 days	mg %	Last 5 days	mg %
Girls														
1946-47														
M.G.	1.01 ± 0.17		1.13 ± 0.03		0.99 ± 0.10		0.86 ± 0.07		0.80 ± 0.05		0.92 ± 0.06		1.04 ± 0.10	
W.H.	0.61 ± 0.11		1.11 ± 0.11		0.96 ± 0.07		0.81 ± 0.05		0.95 ± 0.06		0.96 ± 0.02		0.99 ± 0.01	
B.R.	1.00 ± 0.12		1.15 ± 0.04		0.97 ± 0.03		0.91 ± 0.07		0.96 ± 0.02		0.98 ± 0.04		0.82 ± 0.01	
P.S.	1.08 ± 0.10		1.21 ± 0.06		1.07 ± 0.08		0.83 ± 0.08		0.90 ± 0.05		0.98 ± 0.04		0.82 ± 0.01	
1947-48														
N.A.			1.03 ± 0.05	1.02 ± 0.07	0.85 ± 0.07	0.83 ± 0.08	0.91 ± 0.07	0.95 ± 0.06	0.96 ± 0.02	0.99 ± 0.01	0.81 ± 0.05	0.82 ± 0.01	0.86 ± 0.07	0.80 ± 0.05
B.D.			1.18 ± 0.07	1.19 ± 0.06	0.88 ± 0.06	0.90 ± 0.05	0.91 ± 0.07	0.95 ± 0.06	0.96 ± 0.02	0.99 ± 0.01	0.81 ± 0.05	0.82 ± 0.01	0.86 ± 0.07	0.80 ± 0.05
M.F.			1.25 ± 0.12	1.26 ± 0.10	0.97 ± 0.08	0.98 ± 0.04	0.96 ± 0.02	0.98 ± 0.04	0.99 ± 0.01	0.82 ± 0.01	0.86 ± 0.04	0.81 ± 0.04	0.84 ± 0.05	0.68 ± 0.02
R.R.			1.22 ± 0.12	1.27 ± 0.06	1.03 ± 0.01	1.02 ± 0.04	0.67 ± 0.04	0.72 ± 0.07	0.74 ± 0.06	0.74 ± 0.08	0.82 ± 0.09	0.84 ± 0.05	0.84 ± 0.05	0.68 ± 0.02
Boys														
1946-47														
J.C.	0.78 ± 0.07		1.07 ± 0.10	1.01 ± 0.09	0.91 ± 0.06	0.82 ± 0.01	0.80 ± 0.07	0.81 ± 0.04	0.84 ± 0.05	0.84 ± 0.05	0.84 ± 0.05	0.84 ± 0.05	0.84 ± 0.05	0.84 ± 0.05
V.D.	0.39 ± 0.11		1.01 ± 0.09	0.90 ± 0.11	0.82 ± 0.01	0.86 ± 0.04	0.82 ± 0.01	0.82 ± 0.01	0.82 ± 0.01	0.82 ± 0.01	0.82 ± 0.01	0.82 ± 0.01	0.82 ± 0.01	0.82 ± 0.01
J.J.	0.39 ± 0.04		0.90 ± 0.11	0.90 ± 0.11	0.90 ± 0.05	0.90 ± 0.05	0.90 ± 0.05	0.90 ± 0.05	0.90 ± 0.05	0.90 ± 0.05	0.90 ± 0.05	0.90 ± 0.05	0.90 ± 0.05	0.90 ± 0.05
G.S.	0.61 ± 0.13		1.07 ± 0.03	1.07 ± 0.03	0.90 ± 0.05	0.90 ± 0.05	0.90 ± 0.05	0.90 ± 0.05	0.90 ± 0.05	0.90 ± 0.05	0.90 ± 0.05	0.90 ± 0.05	0.90 ± 0.05	0.90 ± 0.05
1917-18														
T.C.			0.80 ± 0.44	0.99 ± 0.20	0.90 ± 0.02	0.90 ± 0.02	0.82 ± 0.09	0.84 ± 0.05	0.84 ± 0.05	0.84 ± 0.05	0.84 ± 0.05	0.84 ± 0.05	0.84 ± 0.05	0.84 ± 0.05
D.E.			0.71 ± 0.32	0.88 ± 0.10	0.67 ± 0.04	0.67 ± 0.04	0.67 ± 0.04	0.67 ± 0.04	0.67 ± 0.04	0.67 ± 0.04	0.67 ± 0.04	0.67 ± 0.04	0.67 ± 0.04	0.67 ± 0.04
W.P.			1.11 ± 0.11	1.12 ± 0.09	0.81 ± 0.04	0.81 ± 0.04	0.81 ± 0.04	0.81 ± 0.04	0.81 ± 0.04	0.81 ± 0.04	0.81 ± 0.04	0.81 ± 0.04	0.81 ± 0.04	0.81 ± 0.04
D.R.			1.01 ± 0.17	1.09 ± 0.06	0.69 ± 0.02	0.69 ± 0.02	0.69 ± 0.02	0.69 ± 0.02	0.69 ± 0.02	0.69 ± 0.02	0.69 ± 0.02	0.69 ± 0.02	0.69 ± 0.02	0.69 ± 0.02

the last day of the saturation period her plasma value was the highest noted. There is a question, therefore, as to whether a further increase would have resulted if the period had been extended.

There was a statistically significant decrease in the plasma ascorbic acid concentration in all subjects when the mean level of ascorbic acid intake was decreased for the third period to 82 mg, which is slightly higher than the 80 mg

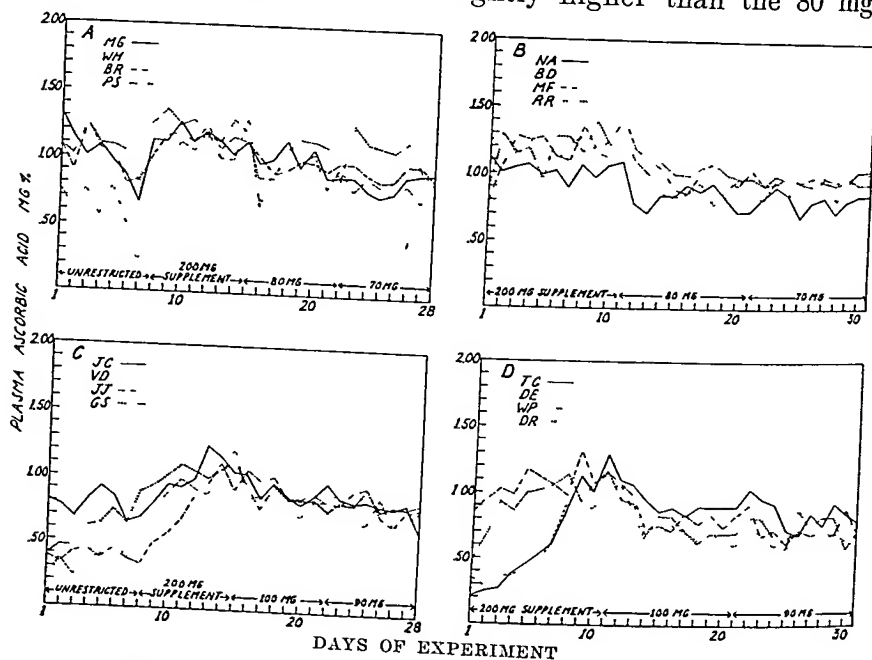


Fig. 1. Daily plasma ascorbic acid values for all subjects.

allowance recommended by the National Research Council. The mean plasma values on this level of intake were very similar to those for each subject during the first or unrestricted period, with the exception of WH, whose value was 0.35 mg% higher than her mean value for the first period. WH's mean plasma value was, however, very similar to those of the other subjects during the third period.

A further statistically significant decrease in plasma ascorbic acid values was shown by two subjects (MG and WH)

during the 4th period when the mean ascorbic acid intake was 71 mg, or 11 mg less than during the third period.

The 10-day saturation period during the 1947-48 study seems to have been sufficiently long for the plasma to become saturated in all 4 girls, as is indicated by a "leveling off" at plasma values above 1.00 mg% (fig. 1B).

A statistically significant decrease in plasma ascorbic acid between the saturation period and the second period, when the mean ascorbic acid intake was 80 mg per day, was shown by all subjects, regardless of whether the plasma values of the last 8 days, or only the last 5 days, of the period were considered.

With these 4 subjects a 10 mg decrease in the level of ascorbic acid intake (to a mean of 70 mg per day) resulted in no statistically significant decrease in the plasma ascorbic acid values when calculated on the basis of the last 5 days, and in only one case (RR) was there a significant decrease when they were calculated on the last 8 days of the period.

### *Boys*

The fairly low mean plasma ascorbic acid values of the boys in the first period of the 1946-47 study, when the ascorbic acid intake was not restricted, were a general indication of previously low intakes (fig. 1C). A marked rise in plasma ascorbic acid was observed in three of the subjects, JC, VD and GS, when 200 mg doses of ascorbic acid were given as supplements during the second or saturation week. Although the mean ascorbic acid intake during this saturation period was very high, 319 to 357 mg per day, it was questionable whether the subjects were "saturated" at the end of the week, particularly VD and GS, whose values were increasing day by day. A steady but more gradual rise was shown by JJ during this period and his mean value for the period was considerably lower than those of the other subjects. On the 4th day of the saturation period JJ reported to the Student Health Service at the College, where he was given a series of injections for poison oak. JJ remained on

the experiment as a matter of interest although his data cannot be considered those of a normal subject and were not included in the statistical analyses.

During the third week 100 mg of ascorbic acid, the recommended allowance of the National Research Council for this particular age, were given to the subjects. A marked downward trend was noticed in the plasma values at this time. The decrease in mean plasma values from the saturation period to the period of the 100 mg mean intake was statistically significant for JC, VD and GS.

In the 4th period, when the ascorbic acid intake was decreased 10 mg below the level of intake during the third period, a further significant decrease in plasma ascorbic acid was shown only by JC. The plasma concentration of GS appeared to have reached a plateau by the end of the period at this level of intake, but whether the plasma values of the other subjects had reached plateaus was not so clearly evidenced.

In the 1947-48 study the concentrations of ascorbic acid in the plasma samples of WP and DR were indicative of saturation by the end of the 10-day saturation period (fig. 1D). At the beginning of the study TC and DE had very low fasting plasma ascorbic acid concentrations, 0.21 and 0.34 mg%, but throughout the saturation period their plasma values continued to rise. The mean value for TC calculated on the data for 8 days was 0.80 mg%, but his mean value was 0.99 mg% when only the last 5 days were considered. For DE a similar difference in the means was observed, i.e., 0.71 mg% on the data for 8 days compared to 0.88 mg% on a basis of 5 days. From the plasma values of the last few days of this period it would appear that these subjects were approaching saturation, but we cannot say that they were definitely saturated.

When the intake of ascorbic acid was decreased to 100 mg in the second period, a statistically significant lowering of the mean fasting plasma ascorbic acid values occurred in all cases when calculated on the basis of the last 5 days of the



experimental period. Calculated on the basis of data from the last 8 days of the period, only two of the subjects (WP and DR) showed a statistically significant decrease in the mean fasting plasma ascorbic acid content. The differences between the means of plasma ascorbic acid values were not statistically significant for TC and DE, the two subjects who started the study with very low values.

A further decrease in the ascorbic acid intake to 90 mg made no statistically significant differences in the mean fasting plasma ascorbic acid concentrations of the 4 subjects when calculated on the basis of the last 5 days of the experimental period. The calculations based on the last 8 days revealed inconsistent results, TC and WP showing a significant decrease between the means whereas DE and DR did not.

In addition to the above-mentioned analysis of the results, the data of both years were also analyzed statistically for variance (Snedecor, '46).<sup>5</sup> A statistically significant difference was found between the means of the saturation period and the period on the recommended allowance of the National Research Council, while the difference in the means between the period on the recommended allowance and the period of 10 mg less than this amount was not statistically significant for the boys but was significant for the girls. It was interesting to note that although the girls were ingesting 20 mg of ascorbic acid less than the boys during comparable periods, they had significantly higher mean plasma ascorbic acid values.

The results of this study were similar to those obtained with the younger adolescent group; that is, the daily allowance of ascorbic acid recommended by the National Research Council did not maintain the plasma values at as high levels as were reached during the saturation period. In 22 of the 24 subjects studied in this laboratory the National Research Council recommended allowance maintained mean plasma

<sup>5</sup> The authors are indebted to Dr. J. C. R. Li, Assistant Professor of Mathematics, for assistance in the analysis of variance.

values of above 0.80 mg% under the conditions of our studies. The two exceptions were older adolescent boys.

#### SUMMARY

1. The recommended allowance of the National Research Council, 100 mg for the 18-year-old boys and 80 mg for the 16- to 19-year-old girls, did not maintain mean plasma values at levels as high as their respective saturation means. For the girls all the mean values were above 0.80 mg%, ranging from 0.83 to 1.07. The boys' values ranged from 0.67 to 0.91 mg%; two out of the 7 values were below 0.80 mg%.

2. When the ascorbic acid intake was decreased to 10 mg less than the recommended allowance of the National Research Council, it was found that for 6 of the 8 girls the 70 mg intake of ascorbic acid was as effective as the 80 mg intake in maintaining the ascorbic acid concentration of the plasma, and that for 6 of the 7 boys an intake of 90 mg of ascorbic acid was as effective as 100 mg in maintaining the plasma ascorbic acid concentration (JJ's values were excluded).

3. The 10-day experimental periods were more desirable than the periods of one week. This was particularly true for the saturation period when some of the subjects had been on diets low in ascorbic acid prior to the study.

4. The data in this study were analyzed statistically by testing the significance of differences between means and by analysis of variance.

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# MAGNESIUM DEFICIENCY IN THE CHICK

## I. CLINICAL AND NEUROPATHOLOGIC FINDINGS<sup>1</sup>

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### EIGHT FIGURES

(Received for publication March 30, 1949)

Magnesium has been shown to be an essential constituent of the diet for the mouse (Leroy, '26), the rat (Kruse et al., '32) and the dog (Orent et al., '32). Almquist ('42) reported that magnesium deficient chicks frequently passed into a convulsive state which sometimes terminated fatally. Duncan et al. ('35) have demonstrated that calves may succumb to magnesium tetany at almost any time after 30 days of age.

When an attempt was made to rear baby chicks on highly purified diets (Bird, '45), a peculiar disorder appeared which indicated that at least one essential nutrient was not adequately supplied by the ration. A subsequent report (Bird, '46) indicated that the symptoms of incoordination and convulsions which were found associated with a cerebellar lesion in chicks resulted from a suboptimum level of dietary magnesium. The present report describes both the syndrome which appeared in the chick and the neuropathology resulting from a deprivation of dietary magnesium.

<sup>1</sup>From a thesis submitted to the Graduate Division of the University of California in partial fulfillment of the requirements for the degree of Doctor of Philosophy granted in June, 1948.

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## EXPERIMENTAL METHODS AND RESULTS

Single-comb white Leghorn chicks were used in these studies. They were removed from the incubators when one day old and placed in electrically heated metal battery brooders equipped with raised wire-screen floors. Either immediately upon hatching or after they were fed a commercial-type chick starter ration for 7 days, the chicks were wing-banded and segregated into experimental groups of equal weight and vigor. The diets and water were fed ad libitum. The composition of the diets and the histological procedures employed are given in the appendix to this paper.

*Clinical symptoms*

Chicks receiving the purified-type of diet, 3A, exhibited symptoms indicative of functional disturbance; i.e., poor growth and feathering, decreased muscle tone, squatting, ataxia, fine palpable tremors, forced movements, progressive incoordination of movements, convulsions, and death.

*Incidence.* Signs indicative of the above condition were not observed in all of the chicks fed diet 3A. The incidence, based upon the number of chicks surviving the experimental period, varied from 63 to 100% (table 1). In the 25 experimental groups, 154 chicks or 93% of the 166 chicks which survived the experimental period exhibited signs of functional disturbances. It can be observed in table 1 that the incidence was not influenced by season. This table also shows that the addition of 0.3% of magnesium sulphate to diet 3A gave complete protection against the appearance of deficiency symptoms.

*Growth.* The data in table 1 indicate that diet 3A had an adverse effect on growth. However, the addition of 0.3% of magnesium sulfate (group 160) produced a growth response, even though it was subnormal.

*Functional disturbances.* Symptoms of functional disturbances first appeared after the chicks had been fed diet 3A for 7 days. It was first noted that these chicks squatted more

TABLE 1

*The incidence of symptoms, the number of survivors, and the growth of groups of chicks fed the magnesium deficient diet and the commercial-type control ration*

GROUP NO.	DIET <sup>1</sup> NO.	CHICKS STARTED			CHICKS SURVIVING THE EXPERIMENTAL PERIOD					
		Date	Age	no.	Total no.	With symptoms		Weight gain /bird		
						no.	%	7 days	14 days	21 days
			days					gm	gm	gm
10	3A	Feb. 1944	7	8	6	6	100	15	18	
12	3A		7	7	6	5	83	13	17	
23	CR		7	7	7	0	0	39	85	
24	3A	Mar. 1944	7	8	6	5	83	11	.	
27	3A		7	8	8	6	75	12		
40	CR		7	7	7	0	0	33	..	
45	3A	April 1944	7	9	8	8	100	33	66	
47	3A		7	10	10	10	100	23	50	
50	3A		7	10	9	9	100	24	47	
62	CR		7	10	10	0	0	40	93	
68	3A	July 1944	7	10	9	9	100	21	41	63
73	CR		7	10	10	0	0	30	80	139
76	3A	Dec. 1944	7	9	8	5	63	16	37	60
81	CR		7	9	9	0	0	31	87	140
84	3A	Feb. 1945	7	10	10	10	100	16	37	
88	CR		7	9	9	0	0	17	56	
93	3A	Aug. 1945	1	10	6	6	100	11	17	
96	3A		1	10	9	9	100	13	21	
101	CR		1	10	10	0	0	10	41	
106	3A	Aug. 1945	7	5	5	4	80	23	43	
111	CR		7	5	5	0	0	22	55	
112	3A	Sept. 1945	7	13	13	13	100	23	50	76
114	3A		7	13	12	12	100	30	59	83
115	CR		7	13	13	0	0	35	75	136
120	3A	Oct. 1945	7	7	7	7	100	25	44	
129	CR		7	7	7	0	0	38	98	
133	3A	Dec. 1945	7	9	7	7	100	22	45	74
135	3A		7	9	8	7	85	29	45	68
140	CR		7	9	8	0	0	29	80	149
141	3A	Feb. 1946	1	16	12	10	83	15	24	28
153	CR		1	16	16	0	0	15	43	95
154	3A	Mar. 1946	1	10	7	6	85	13	12	14
160	3J		1	10	9	0	0	18	38	65
165	CR		1	10	10	0	0	12	39	87
Total	3A			191	166	154	93			
	CR			122	121	0	0			

<sup>1</sup>3A Purified-type of diet deficient in Mg, composition given in appendix.

3J Diet 3A plus 0.3% magnesium sulphate.

CR Commercial-type control ration.

than did those birds fed the commercial-type ration. Shortly thereafter a peculiar gait was observed, which could be characterized by the term "goose-step." This became progressively worse until severely affected chicks could take only a few staggering steps. In handling these chicks, a loss in muscle tonus and the presence of fine, palpable, tonoclonic spasms of the body muscles were noted. Complete paralysis of the leg muscles did not occur, since a strong stepping reflex could be elicited even though a standing position was untenable.

Convulsions were observed in many of the affected birds. These attacks appeared suddenly and unpredictably with no correlation between the severity of the clinical signs and the appearance of convulsions. A bird in a convulsive attack would exhibit propulsive or retropulsive movements, and would fall on its side with its legs fully extended and with its head retracted with or without twisting. If the chick did not succumb during the attack, it relaxed within 10 to 15 seconds and remained in a comatose state for several minutes, after which it recovered. Two or three separate convulsions have been observed in the same bird on successive days.

### *Neuropathology of magnesium deficiency*

*Gross tissue changes.* The chick brains when removed from the cranial fossa showed no evidence of gross pathologic changes.

*Microscopic tissue changes.* Microscopic tissue changes found in the cerebellums of magnesium deficient chicks consisted of degenerative alterations in the Purkinje cells which comprised swelling, nuclear changes, tigrolysis, and alterations in the staining characteristics of the dendrites. No microscopic changes were noted either in the medullary substance or in the cells of the granular and molecular layers. Those portions of the deep cerebellar nuclei visible in these sections were normal.



An increase in the size of the Purkinje cells was one of the striking consequences of magnesium deficiency. This may be noted in plate 1. The diameters of the Purkinje cells were measured in both the normal and deficient chicks (see appendix). The values obtained from these measurements are tabulated in table 2, together with an analysis of variance to demonstrate the effect exerted on them by diet, age, and

TABLE 2

*The mean size and the analysis of variance of Purkinje cells from magnesium deficient and control chicks which have been fed the experimental diets for two- and 4-week periods*

MEAN SIZE				
Chick age	Average diameter of Purkinje cells <sup>1</sup>			
	Diet 3A	Commercial ration		
	$\mu$	$\mu$		
2 weeks	17.1 $\pm$ 1.4	15.2 $\pm$ 1.2		
4 weeks	17.8 $\pm$ 2.0	15.1 $\pm$ 1.4		
ANALYSIS OF VARIANCE				
Source of variance	sq.	df	m.s.	F.
Total	1,113.22	299	3.72	
Between diets	403.07	1	403.07	169.35 <sup>2</sup>
Within diets	710.15	298	2.38	
Between ages on same diet	16.14	2	8.07	3.45
Within ages on same diet	694.01	296	2.34	
Between chicks of same age on same diet	16.13	8	2.01	1.17
Within chicks of same age on same diet	677.88	288	2.35	

<sup>1</sup> Seventy-five cells were measured in each group.

<sup>2</sup> Highly significant at 1% level.

error. As can be seen, there is a measurable increase in the Purkinje cell size in the magnesium deficient animals. The average diameter of these cells was  $2.4\mu$  larger than the average diameter of the normal cells. The analysis of variance clearly indicates that the swelling of the Purkinje cells observed in magnesium deficiency was a true increase, and that this increase was due to the diet fed, since the effects of age and of error were negligible.

Under the low power magnification of the microscope ( $120\times$ ) the Nissl substance in normal Purkinje cells appeared to fill the cell body and to project as a point into the cell dendrites (fig. 1, plate 1). Under higher magnification ( $528\times$ ), the Nissl substance was observed to be composed of discrete bodies (fig. 3, plate 2). These bodies appeared as rounded or elongated or ellipsoid masses which overlapped one another. They were more numerous around the nucleus and in the region adjacent to the cell membrane. A narrow zone midway between the nucleus and the cell membrane was devoid of Nissl substance. The nucleus was capped by Nissl substance, which projected into the dendrites.

Apparent changes in the Nissl substance due to magnesium deficiency could be detected under low power magnification (fig. 2, plate 1). The Nissl substance did not appear as intensely stained as it did in the normal Purkinje cells. This might have been due in part to the swelling, which pushed the Nissl concretions away from one another and brought more cytoplasm into view. That portion of the Nissl substance which normally projected into the dendrites was not visible.

Under higher magnification it was observed that the Nissl substance itself was altered by a magnesium deficient diet (fig. 4, plate 2). Within any one microscopic field, Purkinje cells were observed with the Nissl substance in various stages of disintegration—ranging from a slightly greater than normal dispersion of the tigroid bodies to their complete disappearance. Ghost-like cells were visible which showed only the outlines of the cell and of its nucleus.

A change in the staining characteristics of the Purkinje cell dendrites was noted in the magnesium deficient chick. This change was brought out in sections stained by the Bodian protargol impregnation technique. A photomicrograph of a normal cerebellar section stained by this procedure is shown in figure 7, plate 3, where one may observe the ramification of the Purkinje cell dendrites out to the very edge of the molecular layer. In the cerebellums from the magnesium de-

ficient chicks the Purkinje cell dendrites and their associated climbing fibers were not stained by the protargol impregnation (fig. 8, plate 3), whereas the basket cells and their axons were stained.

Nuclear changes were observed in the Purkinje cells in magnesium deficiency. The Bodian protargol impregnated sections best demonstrated these changes, although they were visible in sections stained with hematoxylin and eosin (figs. 5 and 6, plate 2). In the protargol preparations the normal Purkinje cell nucleus appeared as a slightly ovoid mass of finely granular material which contained one or two nucleoli (fig. 7, plate 3). In contrast, the nuclear material in the magnesium deficient animals contained lighter stained areas, was composed of larger granules and appeared to be pulled away from the nuclear membrane (fig. 8, plate 3).

#### DISCUSSION

The form and character of the Nissl substance have been used in the diagnosis of neuropathologic processes ever since Nissl ('03) published his classification of nerve cells based upon Nissl substance morphology. Even though formed Nissl granules have not been satisfactorily demonstrated in living cells (Penfield, '32), and even though the validity of diagnoses based upon changes in Nissl substance morphology has, at times, been seriously questioned, it is generally accepted that changes in the health or vigor of the nerve cells are reflected by changes in the appearance of the Nissl granules. Both Einarson ('35) and Kappers et al. ('36) believe that these granules and their reaction to toxins and fatigue establish the existence of a substance within the living cell, vital to its effective functional activity, which is indicated—although not directly represented—by the stainable Nissl substance of fixed material.

Cowdry (see Kappers et al., '36) has indicated that differences in the size and in the appearance of the Nissl substance is to be expected in similarly fixed material and even in cells side by side in the same material. Differences in the

size of nerve cells of the same type have also been demonstrated. It cannot be said that abnormal Purkinje cells were never seen in the normal cerebellums under discussion, or that Purkinje cells approaching normal limits in size and staining characteristics were never seen in the magnesium deficient chicks. That this is not a serious objection is well exemplified by the measurements of cell size given in table 2. Here it is shown that there was some overlapping of the upper distribution of cell measurements for the normal cells and the lower distribution for the abnormal cells. Their overlapping was of little practical significance, however, since the results obtained by measuring a relatively few numbers of Purkinje cells were highly significant. Differences in Nissl staining were also observed within any one section, supporting Cowdry's observation. In normal cerebellar sections some Purkinje cells were observed in which the Nissl substance was apparently undergoing changes similar to those seen in the magnesium deficient cerebellums. However, these abnormal-type cells seen in normal brains were few in number and scattered. On the other hand, only a few normal-type Purkinje cells were seen in the magnesium deficient animals.

The clinical symptoms observed in the magnesium deficient chicks can be correlated very well with the microscopic pathology. It is well known that cerebellar disease may be manifested by such symptoms as marked disturbance of equilibrium with ataxia, falling and staggering gait, incoordination of movements, tremor and hypotonia (Kappers et al., '36; Tilney and Riley, '38; Larsell, '39). These symptoms of cerebellar disease were observed in the magnesium deficient chicks. The mossy and climbing nerve fibers in the cerebellum, though having a different distribution within this organ, conduct impulses from other parts of the central nervous system to the cerebellar cortex and activate the Purkinje cells. The activated Purkinje cells, in turn, conduct the nervous impulse out of the cerebellar cortex and exert effects on other regions of the central nervous system. Thus, disturbance in Purkinje cell function will affect those regions

of the central nervous system under cerebellar control and will be manifested clinically as cerebellar disease. The alterations in the Purkinje cell structure observed in magnesium deficiency indicated functional impairment of these cells. This functional impairment had necessarily become apparent in the clinical symptoms of magnesium deficiency, signs indicative of cerebellar disease.

#### SUMMARY

The results of clinical and neuropathologic studies of magnesium deficiency in the growing chick have been presented.

Clinically, magnesium deficiency in the chick is characterized by poor growth and feathering, decreased muscle tone, squatting, ataxia, fine palpable tremors, progressive incoordination of movements, convulsions, and death.

A description is presented of the neuropathologic alterations which were found in the cerebellar Purkinje cells of magnesium deficient chicks. These changes were characterized by swelling, by tigrolysis, by nuclear alterations, and by failure of the Purkinje cell dendrites to stain. There was no evidence of gross pathologic changes.

The neuropathology is correlated with the clinical symptoms.

#### ADDENDUM

Since the publication of this complete study in the thesis submitted to the Graduate Division of the University of California, in partial fulfillment of the requirements for the degree of Doctor of Philosophy granted in June, 1948, a report has been published indicating that similar pathologic changes were observed in the cerebellums of magnesium-deficient rats (Barron et al., '49).

#### APPENDIX

*Composition of the diets.* Diet 3A, which supplied only about one-half of the chick's magnesium requirement, was utilized in most of this study. It had the following composition in grams per 100 gm: water-washed casein 22.2, L-arginine monohydrochloride 0.3, glycine 0.9, L-cystine 0.4, calcium gluconate 5.0, cellulose<sup>3</sup> 5.0, soybean oil

<sup>3</sup> Cellu flour purchased from the Chicago Dietetic Supply Company.

3.0, fish oil<sup>4</sup> 0.25, sodium chloride salt mixture<sup>5</sup> 1.0,  $\text{Ca}_3(\text{PO}_4)_2$  3.5,  $\text{K}_2\text{HPO}_4$  1.3,  $\text{KCl}$  0.3,  $\text{MgSO}_4 \cdot \text{H}_2\text{O}$  0.1,  $\text{Na}_2\text{SiO}_3 \cdot 9\text{H}_2\text{O}$  0.25, cholic acid 0.1, choline chloride 0.2 and glucose<sup>6</sup> 56.2. To every 100 gm of the above mixture were added: solubilized liver eluate equivalent to 4 gm of solubilized liver, thiamine hydrochloride 0.5 mg, pyridoxine hydrochloride 0.4 mg, riboflavin 0.5 mg, calcium pantothenate (dextrorotatory) 1.5 mg, nicotinic acid 1.0 mg, 2-methyl-1, 4-naphthohydroquinone diacetate 1.0 mg, alpha-tocopherol 1.0 mg, and biotin<sup>7</sup> 0.01 mg.

The solubilized liver eluate was prepared by extracting 1 kg of solubilized liver<sup>8</sup> 4 times with two-gallon portions of water brought to pH 4 with sulfuric acid. The solids were allowed to separate by gravity and the supernatant fluid was removed by means of a siphon. The clear extract was then treated twice with 250 gm portions of charcoal<sup>9</sup> at pH 4. The charcoal was removed by filtration and eluted with a solution of 60% acetone and 40% water containing 2% concentrated  $\text{NH}_4\text{OH}$ . The removal of the acetone and the concentration of the eluate were effected by distillation *in vacuo*.

*Histological methods.* Individual chick brains were studied histologically. The chicks were killed by decapitation and their brains removed immediately. The brains were exposed by cutting through the external sagittal groove of the skull and chipping away the bone covering the dorsal and lateral surfaces. After the brains were freed from the ventral surfaces of the cranial fossae by sectioning the cranial nerves, they were immediately placed in the fixing solution. During the process of their removal the brains were subjected to the least possible amount of manipulation and were bathed with an 0.85% solution of sodium chloride to minimize dehydration effects.

In the early phases of this study, Bouin's fluid was employed routinely as a fixing agent. Later, when sections were to be impregnated with silver, the brains were fixed in the following solution:

<sup>4</sup>Fortified sardine oil containing 3,000 I.U. of vitamin A and 400 A.O.A.C. units of vitamin D per gram.

<sup>5</sup>The sodium chloride salt mixture contained 96.471% sodium chloride, 0.49% manganese, 0.1% iron, 0.5% copper, 0.05% zinc, 0.05% aluminum, 0.002% cobalt, and 0.04% iodine.

<sup>6</sup>Cerelose.

<sup>7</sup>The crystalline biotin was donated by Merck and Company, Inc.

<sup>8</sup>Liver fraction "L" donated by Wilson Laboratories, Chicago, Illinois.

<sup>9</sup>Nuchar "C-45" obtained from the Industrial Chemical Sales Co., Covington, Virginia.

37-40% formaldehyde (U.S.P.) 100 ml, distilled water 900 ml, sodium chloride 8.5 gm, and magnesium carbonate 1.0 gm.<sup>10</sup>

The fixed material was embedded in paraffin and parasagittal sections of the brain were cut at 8  $\mu$  to include regions of the cerebral cortex, cerebellum, pons, and medulla oblongata.

These sections were stained with hematoxylin and eosin and with toluidin blue by the procedure recommended by McClung ('29). In later studies, sections were also stained by a modified<sup>11</sup> Bodian protargol impregnation technique (Bodian, '36, '37).<sup>12</sup>

The measurements of the Purkinje cells were made on toluidin blue stained sections with a calibrated Spencer ocular drum micrometer. Twelve slides were examined. These included three slides selected at random from each of the following 4 dietary groups: (a) chicks fed diet 3A for two weeks; (b) chicks fed the commercial-type ration for two weeks; (c) chicks fed diet 3A for 4 weeks; and (d) chicks fed the commercial-type ration for 4 weeks. The diameters of 25 consecutive Purkinje cells were measured. The cell diameter parallel to the boundary between the molecular and granular layers was chosen to represent the diameter of the cell because the point of attachment of the dendrite with its cell body could not always be seen, and thus it was not possible to fix accurately the true cell orientation. It was recognized that these measurements did not necessarily represent the true size of the cell. However, since all of the measurements were consistently made in the same way, their use for comparative purposes was considered to be valid.

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<sup>10</sup> Lyons, W. R. Personal communication.

<sup>11</sup> See footnote 10.

<sup>12</sup> The author wishes to express his appreciation to Mr. Cecil A. Gunns of the Division of Poultry Husbandry for sectioning and preparation of the slides from the fixed material.

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## PLATE 1

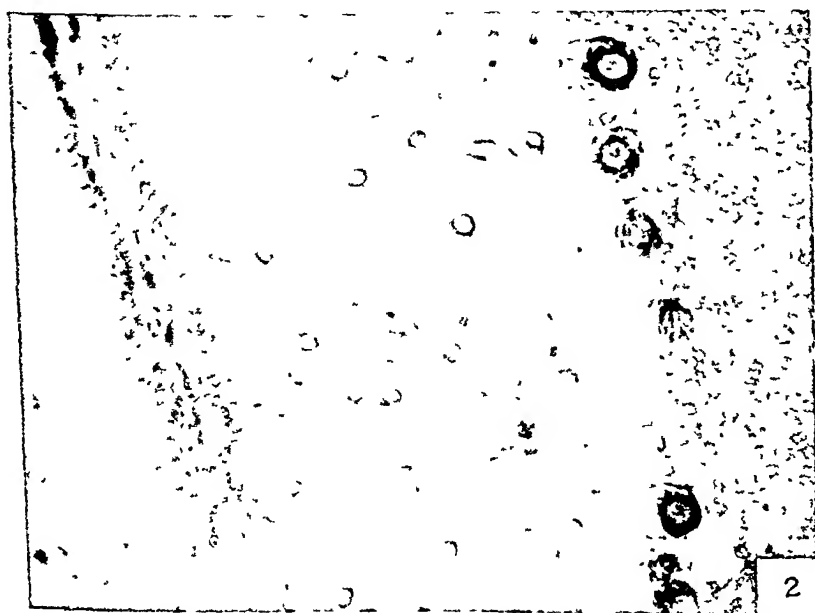
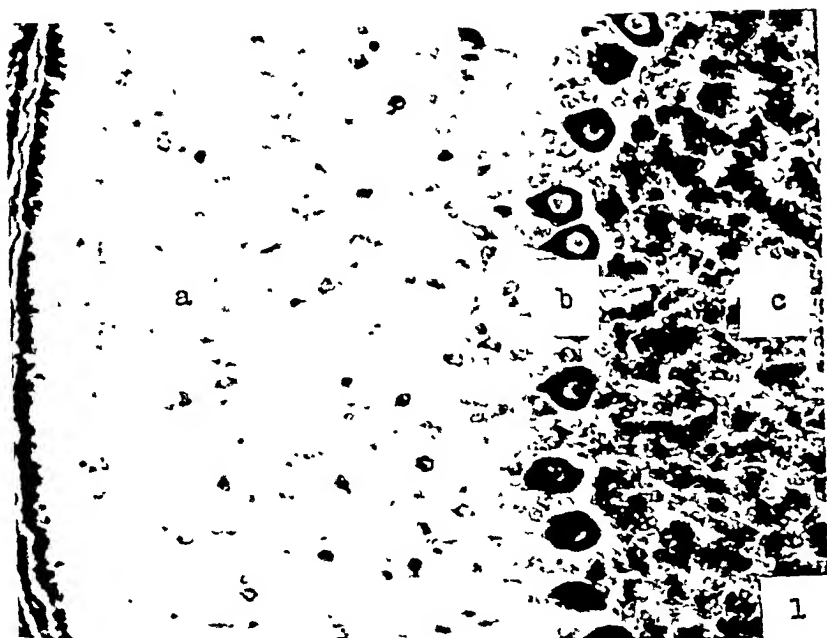
### EXPLANATION OF FIGURES

Photomicrographs of parasagittal sections cut from paraffin embedded chick cerebellums. (a) Molecular layer, (b) line of Purkinje cells, (c) granular layer. Stain, toluidin blue,  $\times 315$ .

1 From chick no. 2757 fed the commercial-type control ration. The Purkinje cells are of normal size and their Nissl substance has a normal distribution within the cells.

2 From chick no. 2676 fed the magnesium deficient diet 3A. The Purkinje cells are swollen and their Nissl substance shows chromatolysis.





## PLATE 2

### EXPLANATION OF FIGURES

Photomicrographs of Purkinje cells from normal and magnesium deficient chicks,  $\times 1100$ .

3 From chick no. 2604 fed the commercial-type control ration. The cells are of normal size. The Nissl granules are normal in appearance and have a normal distribution within the cell. Stain, toluidin blue.

4 From chick no. 2446 fed the magnesium deficient diet 3A. The cells are swollen. The Nissl granules show chromatolysis and have an abnormal distribution within the cells. Stain, toluidin blue.

5 Normal cells seen in chick no. 2604 fed the commercial-type control ration. The nuclear material has a normal appearance. Stain, hematoxylin and eosin.

6 Abnormal cells seen in chick no. 2446 fed the magnesium deficient diet 3A. The cells are swollen. The nuclear material has lost its connection with the nuclear membrane. Stain, hematoxylin and eosin.



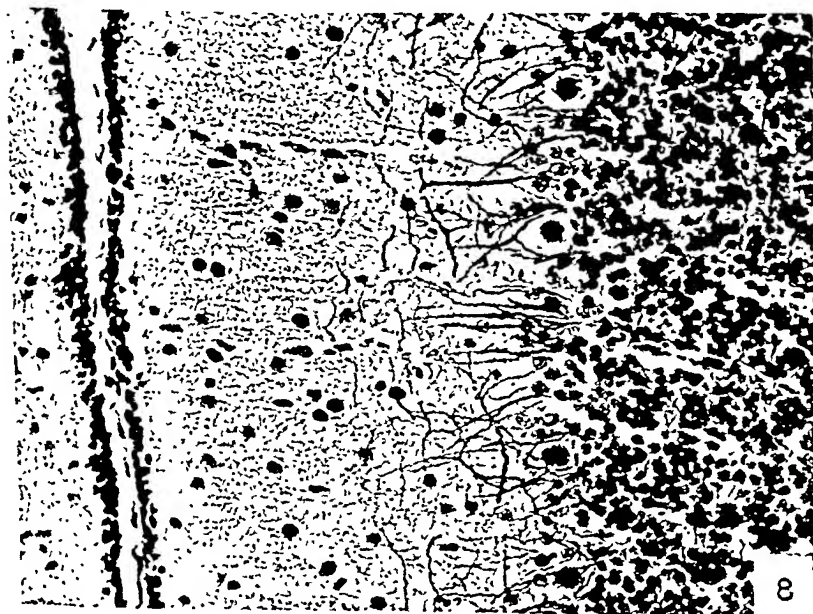
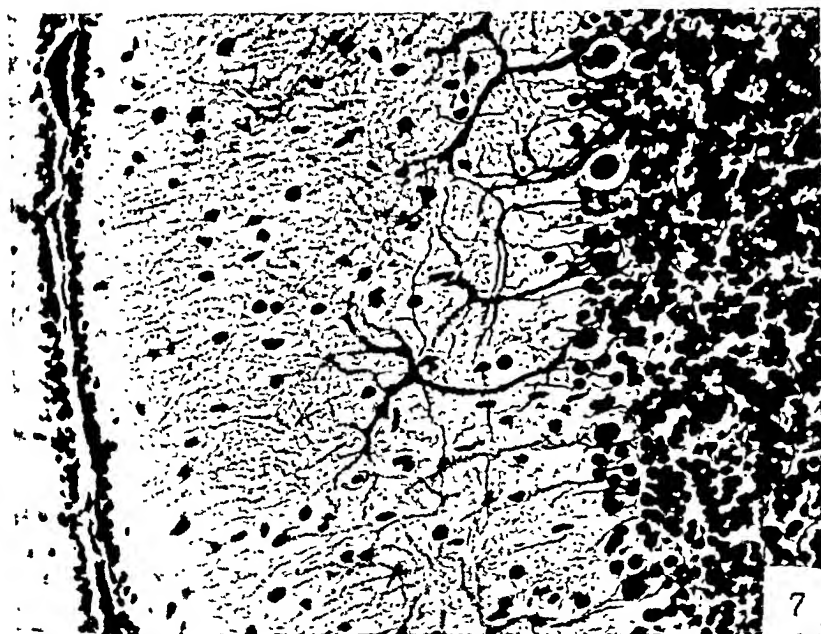
## PLATE 3

### EXPLANATION OF FIGURES

Photomicrographs of parasagittal sections cut from paraffin embedded chick cerebellums stained by the Bodian protargol impregnation technique,  $\times 315$ .

7 From chick no. 3620 fed the commercial-type control ration. The Purkinje cells are of normal size and their dendrites and nuclei show normal staining characteristics.

8 From chick no. 3579 fed the magnesium deficient diet 3A. The Purkinje cells are swollen and their dendrites fail to stain. The Purkinje cell's nuclei contain light staining areas and the nuclear material appears to be separated from the nuclear membrane.





# A PHYSIOLOGICAL AND CYTOCHEMICAL STUDY OF THE KIDNEY AND THE ADRENAL CORTEX DURING ACUTE CHOLINE DEFICIENCY IN WEANLING RATS<sup>1</sup>

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TWENTY-ONE FIGURES

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The occurrence of renal lesions in weanling rats fed a diet deficient in choline was first reported by Griffith and Wade ('39). Degeneration of the kidney tubules associated with cortical hemorrhage developed in these animals after a few days on a deficient diet and could be prevented by amounts of choline too small to influence the deposition of fat in the liver. Spontaneous repair of the kidney damage occurred, furthermore, without the addition of choline to the diet if the animals survived the period of severe uremia (Griffith, '40). Griffith and Wade noted that the renal lesion was accompanied by acute involution of the thymus and suggested that this phenomenon could be the result of a generalized adaptive reaction to stress (the "alarm reaction" of Selye). In the present paper studies are reported on the interrelationships of the kidney and adrenal cortex during the course of acute choline deficiency in weanling rats.

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In the rat it appears that the zona fasciculata of the adrenal cortex secretes hormones of the corticosterone type ( $C_{21}$ -oxy steroids concerned with carbohydrate and protein metabolism) and that the zona glomerulosa secretes hormones of the desoxycorticosterone type ( $C_{21}$ -desoxy steroids concerned with salt metabolism and renal function). During the typical adaptation reaction only the fasciculata of the adrenal cortex is affected (Deane and McKibbin, '46; Deane and Shaw, '47). It became of interest, therefore, with regard to choline deficiency in young rats, to discover (1) the relative response of the two zones of the cortex to acute renal damage; (2) the time relation between changes in the adrenal cortex and the kidney as a guide to determining cause and effect; and (3) the degree of correlation between histological changes in the kidney and changes in kidney function during the development of the renal lesion.

The general plan of these experiments was to kill choline-deficient rats at intervals during the development and regression of the renal lesions. In addition, some of the deficient animals received daily injections of desoxycorticosterone or whole adrenal extract in order to reveal whether or not hormone supplements would alleviate the kidney damage. Changes in the microscopic appearance of the adrenals and kidneys; in the levels of blood non-protein nitrogen, serum sodium and potassium, urinary sodium, potassium, creatinine and glycoeyamine; and in the respiration of kidney slices were determined.

#### MATERIAL AND METHODS

Weanling male rats of the Sprague-Dawley and Long-Evans strains, weighing between 35 and 45 gm, were used in these experiments. No significant differences in the susceptibility of the two strains to choline deficiency were observed. The rats were divided into experimental and control groups, and the deficient animals were fed the following purified diet:<sup>2</sup> al-

<sup>2</sup> Our thanks are due Merck and Co., Inc., Rahway, N. J., the Corn Industries Research Foundation, New York, and the Sheffield Farms Co., Inc., New York, for generous supplies of materials used in the diets.



cohol-extracted peanut meal 20% ; casein 6% ; dextrose 64.8% ; salts (Hegsted et al., '41) 4% ; cod liver oil 2% ; and corn oil 3%. Crystalline vitamins of the B complex were added in the following amounts per 100 gm ration: thiamine 400  $\mu$ g, riboflavin 800  $\mu$ g, nicotinic acid 2 mg, pyridoxine 400  $\mu$ g, and calcium pantothenate 2 mg. The control rats were fed the same diet with choline chloride added at a level of 0.3%. This diet supplies 0.3% methionine, 0.5% cystine, 0.2% total organic sulfur, 0.26% sodium and 0.72% potassium. Growth rates and food consumption were recorded in all experiments. In experiments 2 and 3 (see below) the rats were maintained in metabolism cages with outside feeders and water bottles, and continuous urine collections were made. The urinals were 9-inch evaporating dishes which were screened to separate feces from the urine.

Three experiments were performed. In experiment 1, which lasted 13 days, 24 deficient and 10 control rats were used. Half of the deficient and half of the control rats were given subcutaneous injections of 1 cm<sup>3</sup> of aqueous beef adrenal extract<sup>3</sup> in two divided doses each day. This quantity of hormone will sustain growth in immature adrenalectomized rats (Olson et al., '44). Animals were killed every other day. Experiment 2 lasted 13 days also. Twenty-four rats were used, of which 16 received the deficient diet and 8 the control diet. Half of the experimental and half of the control animals received 0.5 mg desoxycorticosterone acetate in oil once daily. This amount of hormone will more than sustain normal growth in immature adrenalectomized rats (Olson et al., '44). Animals were killed at two-day intervals. In experiment 3 the rats were given no hormone treatment. Eighteen rats were started and 9 were killed — two from the deficient and one from the control groups at 18, 42 and 88 days, respectively, after the beginning of the experiment.

The animals were killed by decapitation and the blood was collected in a tube containing oxalate as an anticoagulant.

<sup>3</sup> We are indebted to Dr. Dwight J. Ingle of the Upjohn Co., Kalamazoo, Mich., for supplying us with the adrenal cortical extract.

Body weight and the weights of the two kidneys, the thymus, and the two adrenals were recorded.

The following chemical procedures were employed. Liver fat (total chloroform-soluble lipid) was determined by the method of Channon, Platt and Smith ('37); blood non-protein nitrogen by the method of Folin and Wu ('19); serum sodium and potassium on the Perkin-Elmer flame photometer (model 52) with an internal lithium standard;<sup>4</sup> urinary sodium and potassium on the flame photometer and in some experiments sodium according to the chemical procedure of Butler and Tuthill ('31); urinary creatinine by the alkaline picrate method of Lambert ('45); and urinary glycocyamine by a modification of the procedure of Dubnoff and Borsook ('41).

In experiment 1 the oxygen consumption of kidney slices was measured by the direct method of Warburg, with KOH and filter paper in the center well. The whole kidney was chilled immediately after killing the animal. Slices of kidney cortex, 0.5 mm thick, were cut and placed in chilled, oxygenated phosphate-saline of the following composition: NaCl 0.135 M, KCl 0.004 M,  $\text{CaCl}_2$  0.001 M,  $\text{MgCl}_2$  0.005 M,  $\text{Na}_2\text{HPO}_4 \cdot \text{NaH}_2\text{PO}_4$  0.0075 M, and glucose 0.2%. The pH of this medium was 7.4. The slices were then transferred to Warburg flasks containing 3.0 cm<sup>3</sup> of this same medium, and the contents were equilibrated with O<sub>2</sub>. The cocks then were closed and oxygen consumption was measured for one hour at 37°C. The dry weight of the tissue was determined after desiccation at 110°C. for two hours. The rate of oxygen consumption has been expressed as Q<sub>O<sub>2</sub></sub>, i.e., the number of microliters of oxygen consumed per milligram of dry weight of tissue per hour.

For microscopic study, the adrenal glands and transverse slices of kidney were fixed in 10% neutralized aqueous formalin. Other slices of kidney were placed in a Zenker-formalin mixture. In a few instances slices of kidney were also fixed in formol-calcium and in "weak" Bouin's fluid for the identification of phospholipids (Baker, '46).

<sup>4</sup>Lithium standard: 15 mEq/L of lithium in diluted samples containing 0-1 mEq/L of sodium or potassium.

The blocks fixed in formalin were washed in running tap water for one hour, sectioned on the freezing microtome at  $15\mu$  and prepared by a group of methods used for characterizing lipid substances (Deane, Shaw and Greep, '48; Dempsey, '48). Lipid droplets in the adrenal cortex displayed all of the reactions characteristic of droplets in the steroid-producing glands, i.e., sudanophilia, a positive Schiff reaction without pre-treatment with mercuric chloride, birefringence and a greenish-white autofluorescence. Those occurring in the kidney were sudanophilic only and were therefore judged to be triglycerides.

The slices of kidney fixed in Zenker-formalin were postchromated and embedded in paraffin; sections were cut at  $3\mu$  and stained for 48 hours with Mallory's phosphotungstic acid hematoxylin for mitochondria. In addition, deparaffinized sections of some of the most damaged kidneys were examined under the fluorescence microscope.

Sections of kidney fixed in formol-calcium were postchromated and stained with acid hematein according to the method of Baker ('46). The control preparations, fixed in "weak" Bouin's fluid, were extracted with pyridine before mordanting and staining. Those substances staining in the first preparation but not in the second are considered to be phospholipids. The most conspicuous structures presenting this reaction in kidney are mitochondria.

## RESULTS

Acute choline deficiency, with fatty liver and enlargement and congestion of the kidneys, occurred in all of the experimental animals fed the deficient diet for 5 days or more. Approximately 15% of the experimental animals died during the acute phase of the renal disease. Choline-deficient rats given adrenal hormones (either desoxycorticosterone acetate or whole adrenal extract) showed no detectable differences from untreated deficient rats, and consequently the data for all experimental animals have been pooled for the following descriptions.

*Gross and chemical changes in  
acute choline deficiency*

Although many of the renal changes in choline deficiency have been described by other investigators, a summary of our findings is submitted for the purpose of relating them to the concomitant changes in the adrenal cortex. The kidneys of the deficient rats began to enlarge on the 4th or 5th day and

TABLE 1

*Changes in kidney size, the respiration of slices of kidney cortex and the gross appearance of the kidney in weanling rats fed a choline-deficient diet as compared to their controls (exp. 1)*

DAYS ON DIET	NO. RATS	MEAN BODY WT.	KIDNEY WT., % BODY WT.	KIDNEY Q <sub>O<sub>2</sub></sub> GLUCOSE 11.1 mM/L	RENAL CON- GESTION <sup>1</sup>	RENAL ISCHEMIA <sup>1</sup>
		gm				
0	2	33	1.16	19.4	0	0
3	3	52	1.18	20.5	0	0
5	3	52	1.40	18.7	2+	0
6	3	62	1.55	16.4	2+	0
7	3	51	2.10	11.0	4+	0
9	4	47	2.77	8.6	4+	1+
11	2	63	1.96	10.0	1+	2+
12	2	70	1.63	13.8	0	3+
13	3	72	1.16	13.3	0	2+
11-13 <sup>3</sup>	3	97	1.05	18.1	0	0

<sup>1</sup> The degree of congestion and ischemia was determined by gross inspection of the kidneys.

<sup>2</sup> Indicates that pale areas occurred on the surface of the congested kidney.

<sup>3</sup> This group was fed the control purified diet during the experimental period.

attained a maximum size on the 9th day of 2.74% of body weight (range: 2.24-3.19%). Congestion of the renal cortex and subscapular hemorrhage appeared on the 5th day and increased *pari passu* with kidney enlargement (table 1). After the 9th day the kidneys began to decrease in relative size and became pale and ischemic. Proportional kidney weight was restored nearly to normal in the deficient animals after 13 days of deficiency.

With the onset of renal damage, food consumption declined and the animals lost weight (fig. 1). The weight of the paired adrenal glands increased from an initial 31.2 mg to 46.6 mg per 100 gm rat body weight on the 9th day. (The absolute weights of the adrenals rose from 15.6 mg at zero days to 23.4 mg at 9 days.) Marked thymus atrophy coincided with the greatest enlargement of the adrenals. After the 9th day the weights of both the thymus and the adrenals returned toward normal and by the 18th day had attained sizes proportionately comparable to those of the control rats. Liver fat, initially 4.1% of the fresh liver weight, rose to 21.1% at 5 days, declined to 12.8% on the 11th day, and returned to 21.7% on the 13th day. The transient decline of liver fat during the period of acute renal damage was probably due to the inanition which supervened at this time.

Both water consumption and urine production decreased during the phase of renal damage. The excretion of sodium and potassium reached a minimum at 9 to 11 days (table 2; fig. 2); at the same time the serum levels were increased—sodium from 149 to 155 mEq/L and potassium from 6.3 to 9.0 mEq/L. The excretion of creatinine (expressed in terms of milligrams of creatinine excreted per kilogram of rat per day) declined 35% during the period of acute renal degeneration. The excretion of glycocyamine, on the other hand, showed a phasic variation. As is shown in table 2, the glycocyamine coefficient (milligrams of glycocyamine excreted per kilogram of rat per day) increased in the early stages of the deficiency (zero to 4 days) but declined sharply to about half that of normal during the period of renal failure. Blood non-protein nitrogen began to increase at the onset of enlargement of the kidney and reached its highest value of 285 mg% on the 11th day (fig. 2). All of these indices of renal function had returned essentially to normal by the 13th day of deficiency.

The respiration of slices of kidney cortex *in vitro* remained normal during the first 5 days of the deficiency (table 1). Thereafter there was a marked decrease in oxygen consumption, the  $Q_{O_2}$  declining from a control value of about 19 to

8.6 on the 9th day. During the next few days the oxygen consumption increased but was still significantly below normal on the 13th day when the experiment was terminated.

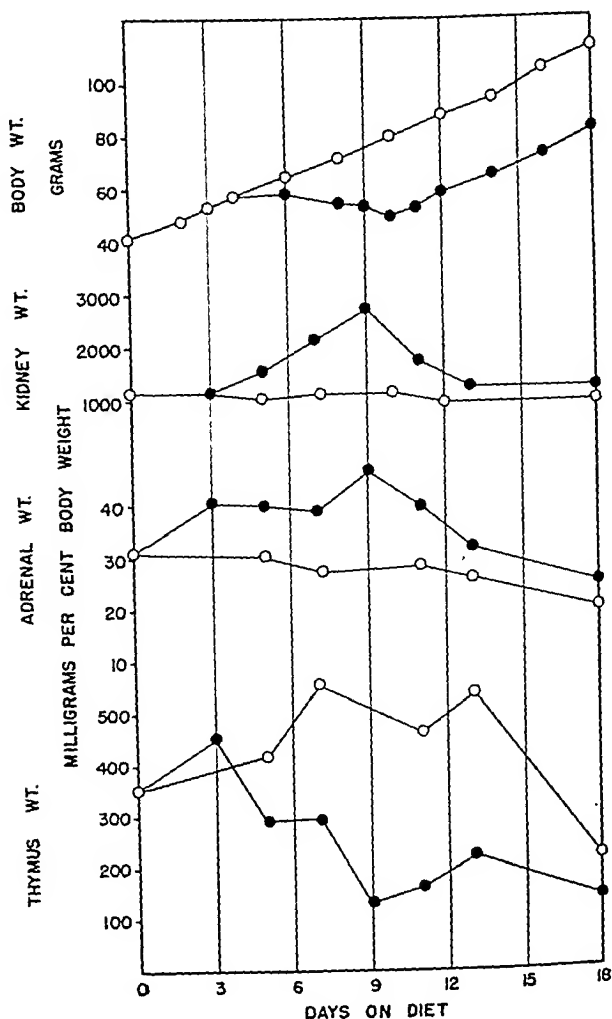


Fig. 1 Changes in body weight and proportional size of the kidneys, adrenals and thymus of weanling rats fed a low choline diet and of their controls. Values for deficient animals, solid circles; values for controls, open circles. Each point represents average data, compiled from all three experiments, for two to 10 rats.

TABLE 2

*Urinary excretion of sodium, potassium, creatinine and glycoeyamine by choline-deficient and control rats at two stages in the experimental period (exp. 2)*

URINARY CONSTITUENT	NO. RATS		VALUES IN MG PER KG RAT PER DAY			
			0-4 DAYS		9-11 DAYS	
	Control	Deficient	Control	Deficient	Control	Deficient
Sodium	10	16	247	258	254	125
Potassium	10	16	988	937	853	398
Creatinine	10	20	36.0	35.8	36.2	23.5
Glycoeyamine	10	20	14.4	27.6	14.1	7.9

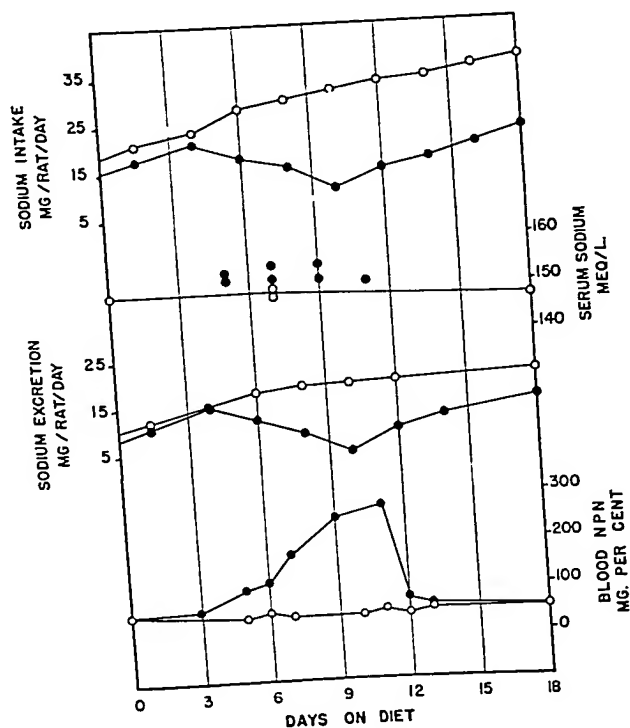


Fig. 2 Changes in sodium intake, serum sodium, sodium excretion, and blood non-protein nitrogen in weanling rats fed a low choline diet and in their controls. Values for deficient animals, solid circles; values for controls, open circles. With the exception of the serum sodium values, which are individual rats, each point represents average data taken from experiments 2 and 3 for two to 10 rats.

*Cytological changes in kidney and adrenal  
in acute choline deficiency*

*Kidney.* Between the third and 5th days small, infranuclear droplets of fat appeared in the tubular cells of the kidney; these were most common in the proximal segments but occurred also in some distal segments. The amount of fat increased rapidly until the 9th day, with both the enlargement of droplets and the involvement of new tubules (figs. 8, 10 and 11, plates 1 and 2). Between the 5th and 7th days congestion of blood appeared in the peritubular plexuses; Hartroft ('48) has demonstrated that this congestion results from blood stasis. By the 9th day hemorrhage beneath the capsule and stasis throughout the cortex were conspicuous (figs. 4, 9 and 11, plates 1 and 2). Following the development of blood stasis, tubular degeneration occurred in the involved areas. Mitochondria disappeared entirely from tubular cells immediately under the capsule where hemorrhage had occurred (figs. 4 and 9), whereas deeper in the cortex mitochondria were still recognizable but appeared fragmented and sparse, as is shown in figures 7 and 8, plate 1. Sections prepared by the Baker method showed a comparable fragmentation and disappearance of the mitochondria. Figures 3 and 6 illustrate the appearance of mitochondria in the normal kidney.

Casts developed in the lumens of the tubules, not only in collecting tubules but in the distal and proximal segments as well (figs. 4, 7 and 8, plate 1). The cast material was only faintly sudanophilic (fig. 11, plate 2), did not contain phospholipids identifiable by the Baker method, and stained with phosphotungstic acid hematoxylin, the color ranging from pink to dark purple. Similar droplets were present in the apical cytoplasm of the cells of the proximal tubules (figs. 8 and 9, plate 1). When unstained sections were viewed under the fluorescence microscope, the casts and intracellular droplets emitted a deep reddish-brown fluorescence similar to that of the static blood; when stained with acid hematein after extraction with pyridine, the cast material was blackened in a fashion identical to the erythrocytes. It seems probable, there-



fore, that the cast material consisted in part of hemoglobin or hemoglobin derivatives.

After the 9th day, the kidney circulation was restored quite suddenly, and the static and extravasated blood promptly disappeared. By the 13th day all cast material had been removed and fat had diminished in all but a few necrotic tubules immediately below the capsule (fig. 12, plate 2). The tubules near the medulla appeared virtually normal but those in the peripheral third of the cortex continued to lack demonstrable mitochondria (fig. 5, plate 1). The microscopic appearance of the kidney was not studied beyond the 13th day of deficiency.

*Adrenal cortex.* The adrenal cortices of rats fed the complete purified diet appeared entirely normal (figs. 13 and 16, plates 2 and 3). In deficient animals the cortex began to broaden on the 5th day and continued to enlarge concomitantly with the increase in kidney weight. This broadening and the increase in adrenal weight mentioned earlier reflected principally a thickening of the fasciculata (figs. 18 and 19, plate 3), although the glomerulosa also became wider. As the fasciculata hypertrophied, the lipid droplets within its cells became small and the birefringent particles uniformly fine (fig. 14, plate 2). This zone returned to normal width on the 11th day.

The changes in the glomerulosa were even more remarkable than those of the fasciculata. By the 5th day the lipid droplets in this zone were greatly reduced in number (fig. 17, plate 3). At 7 days the droplets appearing in this zone were small in size (fig. 18, plate 3). At 9 days, the time of maximum renal enlargement and hemorrhage, the zone had broadened conspicuously and its lipid had all but vanished (figs. 14 and 19, plates 2 and 3). Subsequently small droplets returned (figs. 15 and 20, plates 2 and 3). although the zone remained somewhat thickened through the 18th day. Both the glomerulosa and fasciculata were normal in appearance by the 42nd day of deficiency (fig. 21, plate 3).

## DISCUSSION

*Kidney damage*

The pathogenesis of the hemorrhagic kidney in acute choline deficiency in young rats is not entirely clear, despite the contributions of several investigators (György and Goldblatt, '40; Engel and Salmon, '41; Christensen, '42; Hartroft, '47, '48; and Dessau and Oleson, '47). In reviewing the development of the kidney lesion, it is our purpose to correlate the cytological changes observed by us and by others with changes in renal function and simultaneous changes in the adrenal cortex. All investigators agree that the initial lesion in the kidney cortex is stasis caused by blockage of the capillaries around the tubules rather than by any change in the glomeruli. According to Hartroft, this blockade is caused by fatty infiltration of the tubular cells, in consequence of which they swell and occlude the vessels of the peritubular plexuses. Our observations are entirely compatible with this explanation. Since Patterson, Keevil and McHenry ('44) found a decrease in phosphatide turnover, as measured with  $P_{32}$ , in the kidneys from young choline-deficient rats, the fatty infiltration may result from this decrease in turnover.

Secondary pathological changes develop after stasis and the resultant anoxia have occurred, as evidenced both by the decrease in oxygen consumption of kidney slices *in vitro* and by the fragmentation and even disappearance of mitochondria in the tubular cells. Under conditions of anoxia, fragmentation and dissolution of mitochondria have previously been observed both in kidney and liver cells (Emmel, '40; Deane, Nesbett, Buchanan and Hastings, '47). Furthermore, the hemorrhage and stasis apparently result in hemolysis and the passage of hemoglobin into the lumens of the tubules, where it forms casts. A similar mechanism for cast formation has been described in other experimental situations by Monke and Yuile ('40) and Oliver ('44). Droplets of cast material in the supranuclear cytoplasm of proximal tubule cells are believed indicative of resorption (Gérard, '36; Smetana and Johnson, '42).

During the period of stasis and hemorrhage, renal function is markedly depressed, as indicated by oliguria, nitrogen retention, and a decrease in the excretion of sodium, potassium, creatinine and glycocyamine. With greatest kidney damage the creatinine excretion fell 35%. Since the rat excretes creatinine by way of the glomeruli (Friedman, '47), and since the excretion is unaffected by starvation or lack of dietary methyl groups (Olson, Eder and Stare, '47), this reduction in creatinine excretion suggests a decrease in effective filtration. A similar conclusion may be drawn from the changes in the excretion of electrolytes. The excretion of sodium and potassium was depressed by comparable amounts (about 50%), an effect unlike that induced by adrenal hormones. Moreover, in deficient rats given extra desoxycorticosterone acetate (0.5 mg/day), the excretion of sodium was not significantly changed from that of uninjected animals. In addition, Griffith ('41) found a similar decline in the excretion of phenol red, which is excreted chiefly by the tubules. All of these facts are consistent with the view that in acute choline deficiency whole nephrons are inactivated as a result of the blood stasis.

The excretion of glycocyamine, a compound synthesized by the tubules, was depressed more than was creatinine excretion during the period of maximum kidney damage. The studies of Borsook and Dubnoff ('40), Du Vigneaud et al. ('40) and Bloch and Schoenheimer ('41) indicate that glycocyamine is formed in the kidney from arginine and glycine and is then methylated to creatine in the liver. In adult rats deficient in methyl groups but without any detectable damage to the kidney, glycocyamine excretion rises, presumably because of retarded conversion to creatine in the liver (Olson, Eder and Stare, '47). A similar increase in the excretion of glycocyamine occurred in the young choline-deficient rats of the present study before the onset of kidney congestion. During the height of the renal damage, however, the excretion of glycocyamine declined markedly, most likely because of the

damage to the tubular parenchyma and the consequent failure of these cells to synthesize glycocyamine.

A decrease in the respiration of kidney slices followed the development of blood stasis by one or two days, thus paralleling other signs of reduced tubular activity. This decrease in respiratory capacity went hand in hand with fragmentation and destruction of mitochondria in the tubular cells. Even after restoration of the renal circulation and nitrogen clearance, slices of kidney cortex taken from the periphery, where mitochondrial destruction persisted, showed a depressed oxygen consumption. Mitochondria have been found to contain several important oxidative enzymes, such as cytochrome C, cytochrome oxidase, succinoxidase, and fatty acid oxidase (Lazarow, '43; Hogeboom, Claude and Hotchkiss, '46; Kennedy and Lehninger, '48). It is not unexpected, therefore, that the dissolution of mitochondria in the cells of the renal tubules is associated with a decline in oxygen consumption of slices *in vitro*. In the early phases of this work it was thought conceivable that severe lack of choline might directly interfere with the formation of mitochondria, since the latter contain considerable amounts of phospholipid (Lazarow, '43). It turned out, however, that the dissolution of mitochondria followed instead of preceding blood stasis. Nevertheless, other workers have demonstrated that choline-deficient rats display a decreased respiration in tissues not suffering from blood stasis (liver, Welch, Irving and Best, '35; striated muscle, Abdon and Borglin, '46). In addition, György ('47) has reported tubular nephrosis which was not secondary to kidney congestion in adult rats fed a choline-deficient diet.

### *Changes in the adrenal cortex*

As regards the interrelationships of the kidney and the adrenal cortex in this syndrome, changes in adrenal weight and adrenal cytochemistry paralleled the changes in kidney size and histology during the development and resolution of the renal lesion. No abnormalities of the adrenal cortex

persisted after the recovery of the kidney, despite continued fatty infiltration of the liver. On the other hand, the administration of neither desoxycorticosterone acetate nor whole adrenal extract had any effect on the progress of the renal lesion. It seems clear, therefore, that adrenal insufficiency does not play a rôle in the etiology of the kidney damage, but that the changes in the adrenal cortex are secondary to the renal changes.

Both the zona glomerulosa and the zona fasciculata appeared hyperactive at the time of maximum renal damage, although the glomerulosa appeared far more stimulated. The enlargement of the fasciculata and reduction in the size of its lipid droplets, accompanied by marked atrophy of the thymus gland, suggest an increased secretion of 11-oxy-corticosteroids during the acute phase of the deficiency (Dougherty and White, '45; Deane and Shaw, '47; Bergner and Deane, '48). The immediate recovery of the fasciculata and the thymus upon restoration of kidney circulation and normal food intake, however, together with the relatively mild depletion of fasciculata lipids during the phase of renal damage, suggest that the changes in the fasciculata were referable to the "alarming" stimulus of general inanition and uremia. This stimulation of the fasciculata and the decline in liver fat appear to have been the only changes referable to inanition. Contrary to the conclusions of Christensen and Griffith ('42), there is no evidence of a direct effect of choline deficiency on the pituitary-adrenal-thymus apparatus as in the case of pantothenic acid and thiamine deficiencies (Deane and McKibbin, '46; Deane and Shaw, '47).

The marked hyperactivity of the zona glomerulosa during the period of renal damage, a reaction not noted in other conditions of physiological stress, is the outstanding feature of the adrenal response in acute choline deficiency of young rats. Both hypertrophy of the zone and hypersecretion, as indicated by complete disappearance of lipids (Deane, Shaw and Greep, '48), occurred at the time of maximum kidney

damage. Furthermore, recovery was somewhat slower in this zone than in the fasciculata.

The question of the mechanism by which the kidney damage stimulates secretion by the glomerulosa is of some interest. The glomerulosa, unlike the fasciculata, appears to be independent of pituitary control in the rat (Deane and Greep, '46), and it is believed to secrete salt-regulating hormones of the desoxycorticosterone type (Greep and Deane, '47; Nichols, '48; Deane, Shaw and Greep, '48). Deane, Shaw and Greep have shown that the zona glomerulosa of the rat becomes hyperactive with either sodium deficiency or potassium excess, i.e., whenever there is a decrease in the ratio of sodium to potassium in the blood plasma. During the acute phase of choline deficiency there is a fall in the ratio of sodium to potassium in the plasma, despite absolute increases in both. It seems possible, therefore, that this shift might constitute the stimulus for increased glomerulosa activity.

At the present time, of course, other causes for the stimulation of the glomerulosa cannot be ruled out. That the high level of non-protein nitrogen was not the cause is suggested by the fact that the nitrogen retention persisted after the zona glomerulosa had begun to recover (11 days). Moreover, in several rats made acutely uremic either by removal of the kidneys or ligation of the renal arteries, no changes in the glomerulosa were demonstrable. It remains possible, however, that some humoral product of the damaged kidney could stimulate the glomerulosa.

#### SUMMARY

The effects of acute choline deficiency in weanling male rats on the cytochemistry of the adrenal cortex and the cytology and function of the kidney have been studied.

The renal lesion of acute choline deficiency is characterized by fatty infiltration of the tubular epithelium, congestion and hemorrhage of the cortex, fragmentation and disappearance of mitochondria in the tubular epithelium, and a decrease in the respiration of slices of kidney cortex *in vitro*. At the time

of maximum kidney damage (9 days), casts of material resembling hemoglobin appear in the lumens of the tubules.

Concomitant with the renal congestion there is nitrogen and electrolyte retention. The excretion of glycoeyamine is depressed more than is the excretion of creatinine.

The renal damage is accompanied by an enlargement of the adrenal cortex and atrophy of the thymus. The zona fasciculata appears only moderately stimulated, apparently as a result of the "alarming" stimulus of inanition. The zona glomerulosa is much more markedly stimulated, possibly as a result of altered electrolyte balance during renal failure.

With kidney repair, which occurs without added choline, the adrenal cortex returns to normal, the zona fasciculata recovering more rapidly than the zona glomerulosa. Both zones appear normal by the 42nd day of deficiency, despite the continued fatty liver.

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## PLATE 1

### EXPLANATION OF FIGURES

All figures on this plate are from kidneys fixed in Zenker-formalin solution and postchromated. Paraffin sections were cut at  $3\mu$  and stained with Mallory's phosphotungstic acid hematoxylin for 48 hours. Figures 3 to 5, photomicrographs taken with combined green and yellow filters,  $\times 45$ . The preparations were stained and photographed identically. Figures 6 to 9, drawings made with the aid of a camera lucida,  $\times 90$  objective,  $\times 10$  ocular;  $\times 1690$  at the plane of the drawing.

3 Normal kidney, showing cortex and part of the medulla. The proximal convoluted tubules stain darkly, principally because of their many, highly chromophilic mitochondria (fig. 6). The distal convoluted tubules stain more lightly, and the collecting tubules and Henle's loops only faintly.

4 Kidney of a rat fed the choline-deficient diet for 9 days. Underneath the capsule hemorrhage (H) has occurred; a considerable amount of stagnant blood (S) is visible between the tubules in the outer half of the cortex. Here the tubules no longer stain normally, whereas they stain almost normally in the inner part of the cortex (compare figs. 7, 8 and 9). Many tubules contain homogeneous chromophilic casts: generally these stain purple, sometimes pink. The glomeruli appear normal.

5 Kidney of a rat fed the choline-deficient diet for 13 days. All extravasated and stagnant blood has disappeared, as have the casts. Immediately underneath the capsule the tubules appear collapsed and mitochondria have not reappeared. In the inner part of the cortex the tubules stain in a nearly normal fashion.

6 Normal kidney showing a proximal tubule on the left and a distal tubule on the right. In the cells of the proximal tubule, the long filamentous mitochondria are oriented perpendicularly to the basement membrane and the cytoplasm is quite chromophilic. In the distal tubule the mitochondria are sparser and less well oriented.

7 Kidney of a rat fed the choline-deficient diet for 9 days. Deep portion of cortex, illustrating a proximal tubule on the left and a distal one on the right. Stagnant blood within the capillaries. The mitochondria in the proximal tubule are sparser than normal, and the cytoplasm is less chromophilic. The mitochondria in the distal tubule stain only faintly. A cast is present in lumen of the distal tubule.

8 Kidney of a rat fed the choline-deficient diet for 9 days. Mid-portion of cortex, showing a collecting tubule on the left, and a proximal tubule on the right. Casts are present in the lumens of both. The cells of the proximal tubule contain unstained fatty spaces with a few, light-staining mitochondrial fragments between them. The larger chromophilic spherules in these cells stain in the same fashion as the cast material.

9 Kidney of a rat fed the choline-deficient diet for 9 days. Subcapsular region of cortex, illustrating a proximal tubule surrounded by stagnant blood. The tubule appears collapsed and the cells completely distorted by large chromophilic inclusion bodies. The faintly staining granules within their cytoplasm may be remnants of mitochondria.



## PLATE 2

### EXPLANATION OF FIGURES

All figures on this plate are photomicrographs of organs fixed in 10% formalin for at least two days and then sectioned at  $15\mu$  on the freezing microtome,  $\times 90$ . Figures 10 to 12 are of kidneys, stained with Sudan black B for fatty substances, and photographed with a blue filter. Figures 13 to 15 are of adrenals, photographed under the birefringence microscope; the margin of the capsule has been drawn in by hand.

10 Kidney of a rat fed the choline-deficient diet for 7 days. Droplets of fat (F) stained black with the Sudan dye occur at the bases of the cells in many of the convoluted tubules.

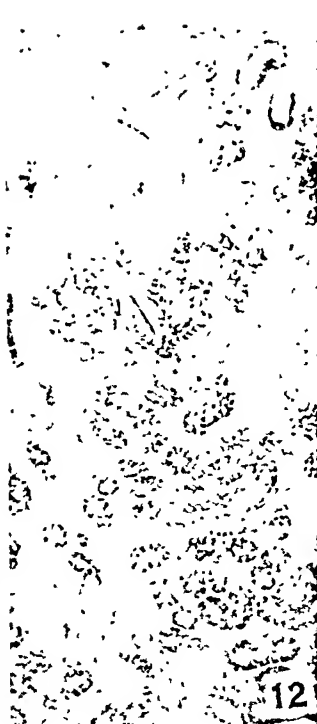
11 Kidney of a rat fed the choline-deficient diet for 9 days. Considerable hemorrhage (H) is visible beneath the capsule. Fat droplets occur in most of the tubules, being particularly concentrated in isolated groups of subcapsular tubules. Gray-staining casts (C) are visible in many tubules. The increase in fat described here was clearly seen in the original material under greater magnification.

12 Kidney of a rat fed the choline-deficient diet for 13 days. Little fat is present except in occasional peripheral tubules. The gray staining of the inner tubules is indicative of mitochondria. The epithelium of some of the outer tubules has collapsed or been sloughed off.

13 Adrenal cortex of a rat fed the control diet for 9 days. Birefringent material characterizes the narrow glomerulosa and the fasciculata. The particles vary in size, some being fine and some coarse.

14 Adrenal cortex of a rat fed the choline-deficient diet for 9 days. The glomerulosa appears broadened. Only occasional, fine birefringent particles are present. The birefringent material in the fasciculata is uniformly fine and is limited to the outer half of the zone.

15 Adrenal cortex of a rat fed the choline-deficient diet for 13 days. The glomerulosa is broad but contains considerable birefringent material, mostly fine. The fasciculata is crowded with birefringent particles, many coarser than in figure 14.



## PLATE 3

### EXPLANATION OF FIGURES

All photomicrographs on this plate are of adrenal glands that were fixed in 10% formalin for at least two days, then sectioned on the freezing microtome at  $15\mu$ . Figures 16 and 21 are from sections stained with Sudan IV and photographed with a blue filter. Figures 17 to 20 are from sections stained with Schiff's reagent and photographed with green and yellow filters combined. All  $\times 90$ .

16 Adrenal cortex of a rat fed the control purified diet for 10 days. The cortex appears normal for a rat weighing 55 gm. Considerable lipid is present in the narrow zona glomerulosa, none in the transitional zone, and a great deal throughout the fasciculata. No zona reticularis occurs in the cortex of the weanling rat.

17 Adrenal cortex of a rat fed the choline-deficient diet for 5 days. Schiff-positive lipid droplets are virtually absent from the glomerulosa. The fasciculata is slightly broadened and its lipid material reacts intensely with the Schiff reagent.

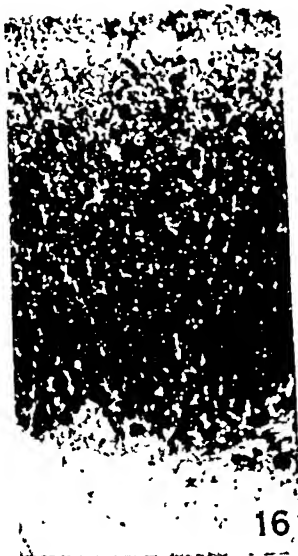
18 Adrenal cortex of a rat fed the choline-deficient diet for 7 days. The lipid droplets in both the glomerulosa and fasciculata are Schiff-positive. The fasciculata is further broadened.

19 Adrenal cortex of a rat fed the choline-deficient diet for 9 days. The glomerulosa appears broad and completely depleted of Schiff-positive material. The fasciculata is extremely broad but not depleted.

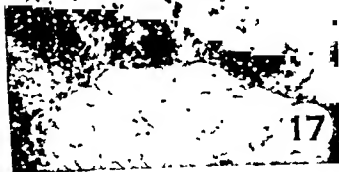
20 Adrenal cortex of a rat fed the choline-deficient diet for 13 days. The glomerulosa remains broad but contains considerable amounts of Schiff-positive lipid. The fasciculata has shrunk to normal size.

21 Adrenal cortex of a rat fed the choline-deficient diet for 42 days. This gland appears normal for a rat weighing 175 gm. Less lipid is present in the inner fasciculata than in the younger rat (fig. 16).

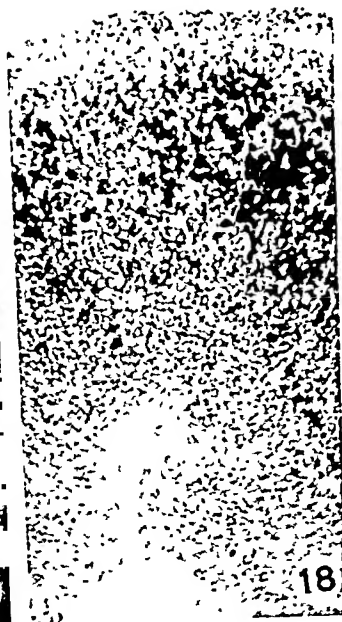
ADRENAL CHANGES WITH RENAL LESIONS  
ROBERT E. OLSON AND HELLEN WENDLER DEASE



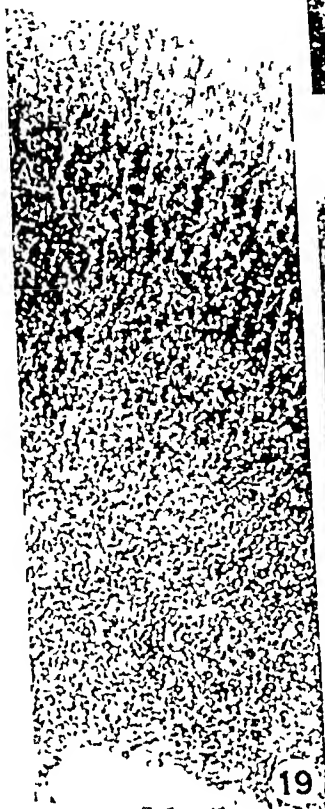
16



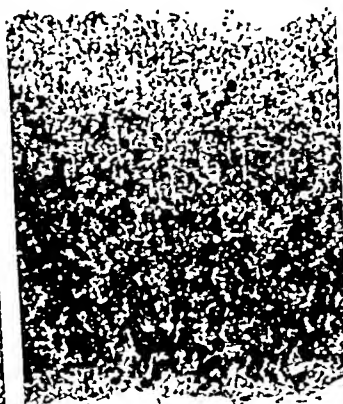
17



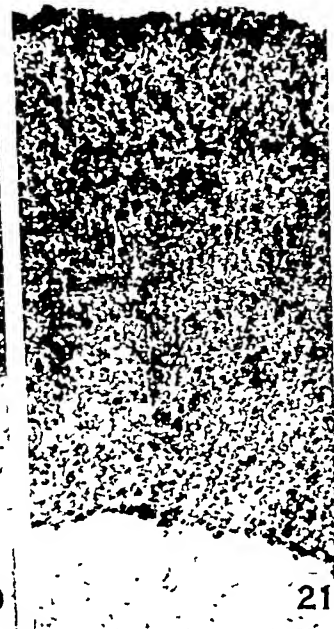
18



19



20



21





# NITROGEN EXCRETION OF WOMEN RELATED TO THE DISTRIBUTION OF ANIMAL PROTEIN IN DAILY MEALS<sup>1</sup>

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Research with animals has shown the inability of the body to store amino acids for use at a later time, and has demonstrated that the body needs to have all the essential amino acids present simultaneously and in adequate amounts for tissue synthesis (Geiger, '47; Cannon et al., '47; Schaeffer and Geiger, '47; Harte, Travers and Sarich, '48). Henry and Kon ('46) found that the supplementary relationship of milk and potato, and of bread and cheese, did not exist if these foods were fed separately on alternate days but could be demonstrated only if the two foods were fed together. Geiger ('48) has shown that diets containing wheat gluten + blood protein, or yeast + blood protein, or yeast + wheat gluten protein promote growth satisfactorily. Growth did not result, however, when these same pairs of proteins were fed separately during alternating 10-hour periods with two-hour intervals between feedings.

The possibility warrants study that the nitrogen metabolism of human subjects may be influenced by the time of in-

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gestion of proteins, especially from the standpoint of the distribution of complete proteins among the usual daily meals. If one meal, such as breakfast, is completely devoid of a high quality complete protein, there is the possibility that the nitrogen utilization of the individual will be impaired even though the total daily intake of protein is adequate.

The study reported here is a preliminary investigation of whether the time element in the feeding of complete animal protein affects the utilization of nitrogen by human subjects. It seemed pertinent to compare under controlled conditions (a) the daily nitrogen metabolism when the amount of protein in the diet was adequate but no animal protein was included in the breakfast, and (b) the nitrogen metabolism on the same protein intake when breakfast included some food which furnished a high quality protein.

#### EXPERIMENTAL

Fourteen college girls volunteered as subjects for this study for 36 days during the summer school session of 1948. They ranged in age from 17 to 23 years, in height from 5 ft. 2 in. to 5 ft. 10 in., in weight from 110 to 157 lb., and in surface area from 1.43 to 1.78 m<sup>2</sup>. The girls lived together in a small dormitory and the research staff supervised the entire regimen. The activities of the different subjects were similar and constant. All were engaged in the sedentary or light work of an intensive school program and those who did not have afternoon laboratories usually had part-time office work.

The 36 days of the study were divided into two 18-day periods, designated as A and B. To facilitate rigid control of the amount and kinds of food and factors such as bulk and rate of digestion, a dietary plan using ordinary foods was made for a three-day period and this plan was repeated 12 times. This three-day diet was planned to meet the recommended allowances of the Food and Nutrition Board of the National Research Council ('48) for all nutrients except protein. Previous study had shown that the usual protein

intake of the subjects was between 60 and 63 gm daily. Because it was desirable not to change the state of protein nutrition of these subjects for a study of only 36 days, the diet was planned to furnish 62 gm of protein daily, 55 of which were from meat, milk, and milk products. During the first 9 days slight adjustments in calorie intake were made to insure that the subjects would remain at constant weight. The adjusted calorie intakes ranged from 1,950 to 2,200 and remained constant for each subject throughout the remaining 27 days of the study.

The difference between period A and period B was in the *distribution* of the animal protein, not in quantity or quality or food source. In period A the breakfast consisted of citrus fruit juice, bread made with water instead of milk, butter, jelly, and coffee or Postum. All of the animal protein was supplied in the noon and evening meals. The diet included 24 oz. of whole milk. During period A, 8 oz. of the milk were served at noon and 16 oz. were served at the evening meal. During period B, 8 oz. of the milk were served at each of the three meals, thus adding animal protein to the breakfast.

Despite rigid control of food selections and preparation the average daily nitrogen intake for period A was 9.78 gm as compared with 10.13 gm for period B; the difference of 0.35 gm was not statistically significant.

During the second 9 days of periods A and B a composite of all the food eaten by each subject at each meal was made, and all excreta for each subject were collected for analysis for nitrogen.

The urine collections for each subject for each 24-hour period were combined according to the times of voiding as follows: after breakfast, up to and including immediately before lunch; after lunch, up to and including immediately before dinner; after dinner, up to and including immediately before retiring at 11 P.M.; rising sample immediately before breakfast. Thus, 4 composites were analyzed for each 24-hour collection for each subject.

Carmine was given in the customary way to mark the stools. Stools for each 9-day period for each subject were combined for analysis.

Nitrogen was determined on acidified urine and on hydrochloric acid digests of food and feces for each subject by the Kjeldahl method.

## RESULTS

The average daily nitrogen excretion for each subject for each period and the differences between the two periods are shown in table 1, together with statistical constants. The

TABLE 1

*Average daily nitrogen excretion of each subject during two 9-day periods*

Average daily nitrogen excretion of each subject during two 9-day periods										
SUBJECT	SURFACE AREA	PERIOD A <sup>1</sup>				PERIOD B <sup>1</sup>				DIFF. IN URINARY EXCRETION IN A AND B
		N excretion			N re-tention	N excretion			N re-tention	
		Urine	Feces	Total		Urine	Feces	Total		
	m <sup>2</sup>	gm	gm	gm	gm	gm	gm	gm	gm	gm
11	1.43	8.58	0.75	9.33	0.45	8.15	0.50	8.65	1.48	0.43
3	1.49	8.16	1.03	9.19	0.59	8.01	0.72	8.73	1.40	0.15
15	1.49	8.86	0.72	9.58	0.20	8.50	0.78	9.28	0.85	0.36
Avg.	1.47	8.53	0.83	9.37	0.41	8.22	0.67	8.89	1.24	0.31
6	1.55	9.26	0.90	10.16	— 0.38	8.11	0.84	8.95	1.18	1.15
1	1.58	8.96	0.71	9.67	0.11	8.27	1.12	9.39	0.74	0.69
13	1.58	9.33	0.82	10.15	— 0.37	8.39	0.59	8.98	1.15	0.94
12	1.60	8.76	0.92	9.68	0.10	8.52	0.93	9.45	0.68	0.24
14	1.62	9.09	0.82	9.91	— 0.13	8.13	0.70	8.83	1.30	0.96
10	1.63	9.22	1.09	10.31	— 0.53	8.26	0.85	9.11	1.02	0.96
5	1.64	9.06	0.84	9.90	— 0.12	7.84	1.15	8.99	1.14	1.22
9	1.65	8.55	0.84	9.39	0.39	8.43	0.86	9.29	0.84	0.12
Avg.	1.61	9.03	0.87	9.90	— 0.11	8.24	0.88	9.12	1.01	0.78
2	1.74	9.54	0.45	9.99	— 0.21	8.74	0.77	9.51	0.62	0.80
8	1.77	9.17	0.64	9.81	— 0.03	9.01	0.77	9.78	0.35	0.16
4	1.78	9.80	0.52	10.32	— 0.54	8.27	1.15	9.42	0.71	1.53
Avg.	1.76	9.50	0.54	10.04	— 0.26	8.67	0.90	9.57	0.56	0.83
Average for all subjects										
	1.61	9.02	0.79	9.81	— 0.03	8.33	0.84	9.17	0.96	0.69
S.D.		0.410	0.171		0.189	0.290	0.190		0.329	
S.E.		0.1097	0.0457		0.050	0.0770	0.0504		0.0878	0.134
<sup>1</sup> The average daily nitrogen excretion of each subject during two 9-day periods										

<sup>1</sup> The average daily nitrogen intake during periods A and B was 9.78 gm and 10.13 gm, respectively.

subjects have been arranged in ascending order of surface area. The total average daily urinary excretion of nitrogen for all subjects for period A was 9.02 gm with a S.D. of 0.41, and 8.33 gm with a S.D. of 0.29 for period B. The difference between the two periods was 0.69 with a S.E. of 0.134, which is highly significant according to the "t" test. Every subject excreted less urinary nitrogen during period B, when animal protein was present in every meal, than during period A when there was animal protein in only two meals. The difference ranged from 0.12 gm nitrogen daily for subject 9 to 1.53 gm for subject 4. The coefficients of variability for all subjects ranged from 5.23 to 16.52 for period A and from 4.24 to 10.14 for period B.

Analysis of the 4 urine composites which were collected for each subject in each 24 hours showed a significantly smaller excretion of nitrogen during the hours from after dinner until bedtime and from bedtime until breakfast time in period B as compared with period A. Analysis of variance for the two 9-day periods for the 14 subjects gave the following F values for the time of voiding stated: morning, 4.05 (significant at the 5% level); afternoon, 1.99; evening, 8.39 (significant at the 1% level); and night, 19.28 (significant at the 1% level).

The average daily fecal nitrogen for all subjects averaged 0.79 gm and 0.84 gm for periods A and B, respectively.

In period A, 8 of the 14 subjects had a total daily nitrogen excretion in excess of the intake; retentions ranged from -0.54 to 0.59 gm daily and averaged -0.03 gm for all subjects.

The situation was different during period B, when some animal protein was served in every meal. The daily nitrogen retention of the subjects ranged from 0.35 to 1.48 gm and averaged 0.96 gm.

The percentage of nitrogen absorbed and retained by each subject during periods A and B is shown in table 2. The average percentage absorption for all subjects was 91.9 for period A and 91.7 for period B. The average percentage re-

tention for all subjects was  $-0.4$  for period A and  $10.3$  for period B.

The relation between the nitrogen metabolism figures and the surface area of the subjects is shown by averages given in tables 1 and 2 for groups of subjects with similar surface areas. There were three subjects with surface areas between  $1.43$  and  $1.49 \text{ m}^2$ ; 8 with surface areas between  $1.55$  and  $1.65 \text{ m}^2$ ; and three with surface areas between  $1.74$  and  $1.78 \text{ m}^2$ . There was no significant difference in the percentage

TABLE 2

*Average daily percentage nitrogen absorption and retention of each subject during two 9-day periods*

SUBJECT	SURFACE AREA	PERIOD A <sup>1</sup>		PERIOD B <sup>1</sup>		DIFF. IN URINARY EXCRETION IN A AND B
		Absorbed <sup>2</sup>	Retained <sup>3</sup>	Absorbed	Retained	
	$\text{m}^2$	%	%	%	%	%
11	1.43	92.3	5.0	95.1	15.4	10.4
3	1.49	89.5	6.7	92.9	14.9	8.2
15	1.49	92.6	2.2	92.3	9.1	6.9
Avg.	1.47	91.5	4.6	93.4	13.1	8.5
6	1.55	90.8	— 4.3	91.7	12.7	17.0
1	1.58	92.7	1.2	88.9	8.2	7.0
13	1.58	91.6	— 4.1	94.2	12.1	16.2
12	1.60	90.6	1.1	90.8	7.4	6.3
14	1.62	91.6	— 1.5	93.1	13.8	15.3
10	1.63	88.9	— 6.1	91.6	11.0	17.1
5	1.64	91.4	— 1.3	88.7	12.7	14.0
9	1.65	91.4	4.4	91.5	9.1	4.7
Avg.	1.61	91.1	— 1.3	91.3	10.9	12.2
2	1.74	95.4	— 2.3	92.4	6.6	8.9
8	1.77	93.5	— 0.3	92.4	3.7	4.0
4	1.78	94.7	— 5.8	88.7	7.9	13.7
Avg.	1.76	94.5	— 2.8	91.2	6.1	8.9
Avg. for all subjects	1.61	91.9	— 0.4	91.7	10.3	10.7

<sup>1</sup> The average daily nitrogen intake during periods A and B was  $9.78 \text{ gm}$  and  $10.13 \text{ gm}$ , respectively.

<sup>2</sup> Calculated according to the formula:  $\frac{\text{gm N intake} - \text{gm N feces}}{\text{gm N intake}} \times 100$ .

<sup>3</sup> Calculated according to the formula:  $\frac{\text{gm N retained}}{\text{gm N absorbed}} \times 100$ .

absorption of nitrogen in either period by the groups with different surface areas. In both periods the urinary nitrogen excretion increased, and the actual nitrogen retention and the percentage retention decreased in step-wise fashion with increasing surface area. For period A the correlation between the surface area and the percentage retention was significant at the 5% level,  $r$  equalling  $-0.62$ , whereas for period B it was significant at the 1% level,  $r$  equalling  $-0.71$ . There were no differences in the metabolism figures which could be related to age.

#### DISCUSSION

The differences in nitrogen metabolism during periods A and B are striking. Significance is added to the differences by the fact that in every case urinary excretion was less and percentage retention was greater in period B than in period A. The similarity in fecal excretion and percentage absorption of nitrogen in period A and period B suggests similar intestinal action during both periods.

If the lower urinary nitrogen excretion and higher retention in period B as compared with period A are considered to indicate better utilization, there was an advantage to these subjects in increasing the amount of protein in the breakfast and providing animal protein at every meal by transferring 8 oz. of milk from the dinner to the breakfast meal.

The present study was planned to simulate the fruit-coffee-roll breakfast and to find an effective yet simple and practical way to improve its contribution to the body's needs. It is for this reason that 8 oz. of milk were used as the addition to the breakfast. There are many factors not included in this pilot study which need to be considered in connection with the distribution of protein in the diet. In the present work the quantity as well as the quality of the protein of the breakfast was increased, although the daily protein intake remained constant. The results might have been different if the protein content of the breakfast had remained constant, or if it had been increased with protein of plant origin. With

lunch at noon and dinner at 6:00 o'clock, all the animal protein was eaten within a period of 6 to 7 hours, leaving a period of 17 to 18 hours when no animal protein was taken into the body. The nitrogen metabolism might have been different if animal protein had been given for breakfast and dinner instead of for lunch and dinner. Because of the time factor, lack of animal protein in the breakfast may impair nitrogen utilization more than its lack in the mid-day meal. Such questions as these are being investigated further.

The question arises as to whether dermal loss of nitrogen should be considered. The average of the mean daily air temperatures in period A was 75°F. and in period B 77°F., which suggests that loss of nitrogen in perspiration would have been similar during the two periods. Mitchell and Hamilton ('49) studied the dermal loss of 6 men under widely varying conditions and found no compensatory relationship between urinary and dermal loss of nitrogen.

It is not within the scope of this study to consider nitrogen requirements. The subjects were young adults who probably had need of some storage of nitrogen. This storage was accomplished during period B, when there was more and better quality protein in the breakfast than during period A. Neither can these findings be compared with other studies on nitrogen metabolism because it has not been customary to describe in the literature details of the amount and quality of protein in each of the daily meals served to research subjects.

In view of the nature and significance of the findings in the present study, it is possible that the distribution of quantity and kind of protein as well as the total daily amount of protein must be considered in determining protein requirements and utilization.

#### SUMMARY AND CONCLUSIONS

The nitrogen metabolism of 14 college-age women on a controlled diet which included no animal protein in the breakfast was compared with their nitrogen metabolism when the



breakfast included 8 oz. of milk, although the total protein intake remained unchanged.

During period A, when no animal protein was included in the breakfast, the average daily urinary excretion for all subjects was 9.02 gm nitrogen, S.D. 0.410, as compared with 8.33 gm, S.D. 0.290, during period B when animal protein from 8 oz. of milk was furnished with the breakfast. The difference in urinary excretion was 0.69, S.E. 0.134. In period A the average daily nitrogen absorption was  $-0.4\%$  as compared with  $10.3\%$  in period B. The average daily nitrogen retention was  $-0.03$  gm in period A as compared with 0.96 gm in period B.

In view of the nature and significance of the findings, it is possible that the distribution of quantity and kind of protein in the three meals, as well as the total daily amount of protein, must be considered in determining protein requirements and utilization.

#### ACKNOWLEDGMENT

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## THE IRON REQUIREMENT OF SIX ADOLESCENT GIRLS

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ONE FIGURE

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Information regarding the iron requirement of adolescent girls is meager. A dietary allowance of 15 mg per day has been recommended for girls 13 to 15 years of age by the National Research Council ('48). The recommendation is accompanied by a statement that "No data are available for the requirement during adolescence. The allowances recommended were estimated on the assumption that needs are greater than those of the adult."

The needs of children for iron differ from those of the adult because of growth. To choose a method which will accurately assess the requirements during growth is difficult. One possible method is to find the lowest iron intake which will support a good hemoglobin level. Since hemoglobin levels respond slowly to changes in the dietary intake, this method requires lengthy periods of study, preferably two or more years, for each level of iron intake. Johnston and Roberts ('42) applied this method to pre-adolescent children on one level of intake.

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Another method was used by Darby et al. ('47), who calculated the daily requirement of absorbable iron for children. The calculations were based on the percentage uptake of radioactive iron and on estimates of needs for storage and growth. This method for determining the dietary requirement depends upon the use of estimates of "absorbable" iron in foodstuffs. The author states: "at present there exists no reliable means of estimating the quantity of absorbable iron in a dietary; hence, the requirement of total dietary iron cannot now be ascertained by this method."

A method used by investigators in early years is the balance study. It was used on the assumption that a child would retain only the iron that he needed and that any excess would be excreted. The dietary level giving maximum retention was then taken to be the level of iron required. That assumption is now known to be erroneous. Once iron is absorbed by the body little is secreted back into the gastrointestinal tract, and the amount retained is not necessarily the amount needed. Thus the balance study in itself is not applicable to finding the iron requirement of children, but it is of value in determining the amount of iron which is retained from a given diet.

The method chosen for the present study had to be such that the needs of the adolescent girl who had reached the menarche would be taken into consideration. She needs to retain iron not only for growth but also for replacement of menstrual losses. If these needs are determined, the balance study can then be used to find the level of iron intake which will provide sufficient retentions to cover her needs.

Since there is no method available at present for determining the amount of iron which needs to be retained for growth, the decision was made to use a value based on estimates made by Heath and Patek ('37). They calculated the requirement of iron for growth on the basis of the annual gains in circulating and non-available tissue (extra-circulating) iron. The amount of iron which needs to be retained for replacement of menstrual losses can be determined directly by analysis of the menstruum.

## PROCEDURE

The subjects were 6 girls, 13 and 14 years of age, all of whom had passed the menarche and were in excellent health; all had a history of good food habits. The age, height, weight and age of menarche of the subjects are given in table 1. They were housed and fed for 9½ weeks in the same building in which the laboratory is located.

Two levels of iron were administered in two consecutive 4-week periods preceded by an adjustment period of one week. During the adjustment period and the first 4-week period, 8 to 9 mg of iron were fed. This level was selected

TABLE 1

*The age, height, weight and the age of menarche of the 6 subjects*

SUBJECT	AGE <sup>1</sup>	HEIGHT	WEIGHT	AGE OF MENARCHE
	<i>years</i>	<i>in.</i>	<i>lb.</i>	<i>years</i>
A	14	64½	102½	13
B	14	62	125½	13
C	13	64½	111½	10
D	14	61½	109½	13
E	13	60½	79½	12
F	13	67½	122½	12

<sup>1</sup> Age to nearest birthday.

on the basis of previous work done on college women in this laboratory (Johnston, Frenchman and Boroughs, '48), in which an intake of 7 mg per day was found to be on the borderline of adequacy for the subjects. The slightly higher level was fed to the adolescent girls on the assumption that they would need more iron than the college woman. In case the first level was on the borderline or too low, a somewhat higher level of 11 to 12 mg of iron per day was chosen for the second 4-week period. One level of calcium, approximately 1 gm per day, was fed during the entire study to eliminate the possibility that a change in the level of calcium might affect the absorption of iron.

The basal diet was planned to be adequate in all known respects and to include a wide variety of natural foods. Two or more servings each of fruits and vegetables, one serving of meat and three glasses of milk for drinking (182 gm each) were included daily. To avoid a high phytic acid level in the diet and yet to include foods customarily eaten, a whole grain breakfast cereal, walnuts, shelled beans, cocoa and chocolate were used only once each week. Enriched bread was included daily in the basal diet.

To take care of differences in appetite and caloric needs, certain foods which contain little or no iron were allowed *ad libitum*. These foods were: unenriched bread; butter; grape jelly; sugar; lemonade made with distilled water; cookies made with unenriched flour, sugar and butter; and candy made from distilled water and other ingredients containing negligible amounts of iron. A limited number of bottles of a low-iron cola beverage were allowed each week. The weights of all foods eaten *ad libitum* were recorded. While these foods were low in iron, the quantity eaten by the subjects made the total iron intake vary somewhat from week to week and from subject to subject.

To plan the diet for the first experimental period during which the food contained 8 to 9 mg of iron per day offered no problem and required only the omission of foods containing extremely high quantities of iron, such as liver. One serving each of spinach, green peas and green beans was included each week. The level of iron was increased to between 11 and 12 mg per day by increasing the size of servings of some foods high in iron such as meat, eggs and vegetables, and by the addition to the original diet of a few foods rich in iron. These foods were dried apricots, dates and a small serving of liver sausage.

The menu was planned on a weekly basis and repeated in successive weeks. The basal diet was carefully controlled so that the level of iron in it would be the same for all 4 weeks. Frozen fruits and vegetables and most of the fresh vegetables were obtained in sufficient quantity at the beginning of the

study to carry through the 9 weeks. Where possible, other items were purchased in one lot for the entire study. All fat was trimmed from the cooked meats and the lean portion only weighed. The foods were prepared and served with particular attention paid to avoiding the possibility of contamination; distilled water was used for cooking and drinking; all cooking utensils were either of aluminum or glass and the kitchen tools were of stainless steel.

Composites of foods in the basal diet were made each week. Aliquot portions equal to one-fifth of the serving weight were composited independently by two people. These were mixed in a mechanical blender, treated with concentrated HCl, heated and bottled. The milk, bread, biscuits, rolls, cola drinks, lemonade, grape jelly and cookies were analyzed separately. The feces were marked by using carmine capsules. Weekly composites of feces were mixed in a blender, acidified with HCl, heated and stored in glass. Urinary collections were made for a weekly period on each level of intake and an aliquot portion was acidified with HCl and stored. Menses were collected during and after the experimental period for a minimum of 4 menstrual periods for each subject. Tampons obtained in one lot were used for collection of the menses. The used tampons were allowed to stand several hours in redistilled water, acidified with HCl, heated to form a slurry and stored in glass. A correction was made for the iron in the tampon. A record was kept of the cycle lengths for each subject.

The foods, feces, urine and menses were each wet-ashed with nitric and sulfuric acid and the nitrosyl-sulfuric acid was hydrolyzed by boiling with water according to the method of Roberts, Beardsley and Taylor ('40). Iron was determined by the thiocyanate method of Stugart ('31), and the intensity of the color read in an Evelyn photoelectric colorimeter.

Venous blood samples were taken three times during the study to determine hemoglobin values, red blood cell counts and serum iron levels. They were taken at 4 P.M., after a period of inactivity to eliminate the possible effects of exercise

on the level of these components of blood. Each determination was made in duplicate. For serum iron 2 ml samples of serum were analyzed; the protein was precipitated by the method of Kitzes, Elvehjem and Schuette ('44) and the iron was determined by the *o*-phenanthroline method of Saywell and Cunningham ('37). Hemoglobin was determined by the method of Evelyn ('36). Cell counts were made in duplicate on blood from each of two pipettes.

## RESULTS AND DISCUSSION

### *Daily intake, retention and absorption*

The average daily intake of iron for the first 4-week period for all the subjects was 8.6 mg, and ranged from 8.0 to 9.6 mg (table 2). On the diet with the higher level of iron, the average daily intake was 11.7 mg and ranged from 10.9 to 13.3 mg (table 2). Subject F, the tallest girl, ate the largest amount of the food allowed *ad libitum* and therefore received the most iron; subject E, the shortest girl, received the least iron because she ate little in addition to the basal diet.

Retentions of iron by the subjects represent the difference between that in the food intake and that in the feces and urine (table 2). On an average intake of 8.6 mg per day, average iron retentions for each subject were 0.80, 0.86, 1.22, 1.23, 1.42 and 1.54 mg per day. When the average intake was 11.7 mg per day, the average retentions for each subject were 0.33, 1.15, 1.54, 1.76, 2.10 and 2.22 mg per day. On the diet containing the larger amount of iron, higher retentions occurred for all except subject B, whose retention was so much lower than that of the other subjects and than her own first period that it appeared to be atypical. No explanation could be found for the sudden drop in her retention from 1.54 mg per day during the first period to 0.33 mg in the second period.

On a percentage basis, the subjects retained 9, 9, 14, 14, 18 and 19% of the iron from the diet with 8.6 mg per day, and 3, 10, 13, 16, 16 and 19% from the diet with 11.7 mg per day. The average percentage retentions for all the subjects were



TABLE 2  
The excretion and retention of iron by 6 subjects on two levels of iron intake

IRON REQUIREMENTS

*The excretion and retention of iron*

SUBJECT	ADJUSTMENT PERIOD	FIRST PERIOD					SECOND PERIOD					
		Week I	Week II	Week III	Week IV	Week V	Average	Week VI	Week VII	Week VIII	Week IX	Average
<b>A:</b>												
Fe in food (mg/day)	8.20		8.06	8.77	8.73	8.36	8.48	11.28	11.78	11.74	11.15	11.49
Fe in feces and urine (mg/day)			7.41	7.47	7.80	8.03	7.68	9.50	10.66	10.67	10.51	10.34
Fe retained (mg/day)							0.80					1.15
<b>B:</b>												
Fe in food (mg/day)	7.98		8.05	8.38	8.14	7.76	8.08	10.77	10.94	11.09	10.62	10.86
Fe in feces and urine (mg/day)			7.42	5.48	5.60	7.66	6.54	10.11	10.78	10.40	10.82	10.53
Fe retained (mg/day)							1.54					0.33
<b>C:</b>												
Fe in food (mg/day)	8.18		8.24	8.77	8.81	8.26	8.52	11.60	11.81	12.41	11.50	11.83
Fe in feces and urine (mg/day)			6.89	7.46	7.14	7.69	7.29	10.29	9.65	10.81	10.40	10.29
Fe retained (mg/day)							1.23					1.54
<b>D:</b>												
Fe in food (mg/day)	8.21		8.27	8.89	9.41	9.29	8.96	11.91	11.92	12.25	11.78	11.96
Fe in feces and urine (mg/day)			6.82	6.60	7.45	10.11	7.74	10.01	9.01	9.93	10.03	9.74
Fe retained (mg/day)							1.22					2.22
<b>E:</b>												
Fe in food (mg/day)	7.89		7.94	8.39	8.08	7.66	8.02	10.82	11.14	11.08	10.62	10.92
Fe in feces and urine (mg/day)			6.15	7.05	7.12	6.08	6.60	9.02	8.87	9.67	9.08	9.16
Fe retained (mg/day)							1.42					1.76
<b>F:</b>												
Fe in food (mg/day)	8.32		8.57	9.51	10.23	10.10	9.60	13.61	13.51	13.59	12.36	13.26
Fe in feces and urine (mg/day)			8.77	8.25	8.90	9.05	8.74	11.18	10.86	12.63	9.99	11.16
Fe retained (mg/day)							0.86					2.10

14 and 13%, respectively, of the iron from the two diets. The unusually low retention of subject B from the higher level of intake lowered the average retention for the second period. The percentage of absorption also was calculated. For this, the iron in the feces was subtracted from that in the food intake. Because the amount of iron in urine is so small, the percentage absorption values were the same as the retention values. These values are similar to those in the literature for girls younger than these and for women. Darby et al. ('47) reported that girls 10 years of age absorbed 14.5% of a test dose of radioactive iron. College women studied by Johnston, Frenchman and Boroughs ('48) absorbed 11% from a normal mixed diet containing 7 mg of iron per day, while adult women studied by Widdowson and McCance ('42) absorbed 12.5% from a diet in which white bread made up 40 to 50% of the calories and the remainder were provided by a mixed diet.

### *Blood picture*

The blood picture of the subjects as indicated by hemoglobin values, red blood cell counts and serum iron levels was satisfactory. Hemoglobin values were 13.2 gm per 100 ml of blood or above, and red blood cell counts were all  $4.35 \bar{M}$  per  $\text{mm}^3$  or above (table 3). These values are above the average reported by Leichsenring, Donelson and Wall ('41) for girls of this age. Serum iron levels were variable, ranging from 57 to 97  $\mu\text{g}$  per 100 ml of serum (table 3). These levels are similar to those observed in the serum of other girls of this age in work done in this laboratory (unpublished). The highly satisfactory blood picture suggests that the 6 subjects were absorbing iron in a normal fashion, since all probably had normal stores of iron.

### *Menstrual losses*

Average menstrual losses for 4 or more periods varied widely among the subjects and ranged from 6.12 to 50.11 mg per period (table 4). Considerable variation in iron losses

per period for each girl was observed (fig. 1). Losses for subject A, for example, ranged from 1.73 to 13.83 mg per period. In 4 of the 6 subjects, the first menses collected during the study had the least iron (fig. 1). Whether or not the unusual situation contingent upon being a part of the experiment somehow affected the menstrual loss during the first collection period is a question. In a search of the literature no studies were found regarding iron losses in the menses of

TABLE 3

*Hemoglobin values, red blood cell counts and serum iron levels for the 6 subjects determined during the last week of each period*

SUBJECT	A	B	C	D	E	F
<i>Hemoglobin in gm per 100 ml of blood</i>						
Adjustment period	13.7	13.4	13.8	13.0	15.2	14.3
8.6 mg-intake period	14.2	13.8	13.6	13.3	14.8	13.9
11.7 mg-intake period	13.3	13.4	13.6	13.2	14.6	14.0
Average	13.7	13.5	13.7	13.2	14.9	14.1
<i>Red blood cell counts in <math>\bar{M}</math> per mm<sup>3</sup></i>						
Adjustment period	4.87	4.35	4.50	4.24	4.88	4.65
8.6 mg-intake period	4.90	4.38	4.43	4.38	4.80	4.38
11.7 mg-intake period	4.66	4.61	4.68	4.42	4.81	4.44
Average	4.81	4.45	4.54	4.35	4.83	4.49
<i>Iron in <math>\mu</math>g per 100 ml of serum</i>						
Adjustment period	105	86	76	58	74	72
8.6 mg-intake period	92	76	75	64	76	70
11.7 mg-intake period	94	79	44	50	85	72
Average	97	80	65	57	78	71

girls 13 and 14 years of age, but iron losses during menstruation have been studied in adult women. Frenchman and Johnston ('49) reviewed the literature on this subject and found that reported iron losses in the menses of 184 women ranged from 2.28 to 78.96 mg per period. The range of iron losses for the 6 girls in this study was from 0.59 to 74.38 mg per period, which was as large as that for the adult women studied by others.

In order to calculate the amount of iron needed daily to replace menstrual losses, information regarding the length of the menstrual cycle is necessary. Considerable variation was observed in the length of the cycles of the present subjects. The average length of the cycle of the 6 girls was 29.8 days and ranged from 21 to 41 days (table 4). The average number of days in these cycles is somewhat less than that reported by Engle and Shelesnyak ('34), who found an average of 33.9 days in the menstrual cycles of 100 young girls.

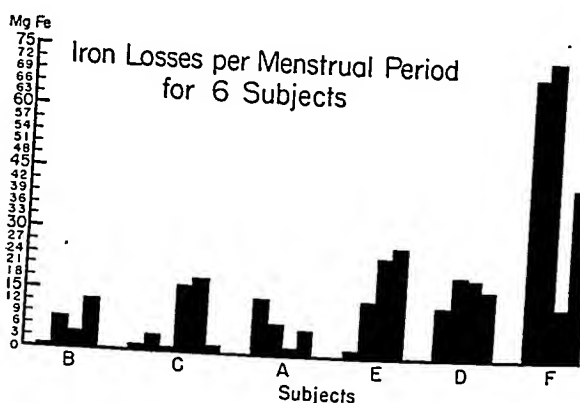


Fig. 1 Each bar represents the milligrams of iron in the menstruum from one menstrual period.

TABLE 4  
*Daily retention of iron required by the 6 subjects for replacement of menstrual losses*

SUBJECT	NO. OF MENSTRUAL PERIODS	LENGTH OF CYCLE <sup>1</sup>		AVERAGE FE LOSS PER PERIOD	DAILY RETENTION OF FE NEEDED FOR REPLACEMENT <sup>2</sup>
		Average	Range		
		days	days	mg	mg
A	4	41.0	35-46	7.46	0.18
B	4	28.0	24-31	6.12	0.22
C	6	21.0	13-31	7.28	0.35
D	4	31.5	29-33	17.78	0.56
E	4	23.3	20-25	16.94	0.73
F	4	33.7	19-43	50.11	1.49
Average		29.8		17.62	0.59

<sup>1</sup> The length of the cycle is the number of days from the beginning of one menstrual period to the beginning of the next.

<sup>2</sup> This value was computed as follows:  $\frac{\text{Average iron loss per period in mg}}{\text{Average length of the cycle in days}}$

According to Reymert and Jost ('47), who studied 100 girls 10 to 18.5 years of age, young girls have more irregular menstrual cycles than do older ones; this irregularity, according to Engle and Shelesnyak ('34), decreases steadily with increasing elapsed time since the menarche. Among the girls in this study, subject F, who had passed the menarche shortly before the beginning of the study, had the most irregular cycles; they ranged from 19 to 43 days in length. In view of the variability observed in the menstrual picture, the menstruum from 4 periods is probably the minimum which should be collected and analyzed from girls of this age to obtain a representative average value. Analysis of the menstruum from more than 4 periods would be highly desirable.

To find the daily retention of iron needed for replacement of menstrual losses, the following formula was applied:

$$\frac{\text{average iron loss per period in mg}}{\text{average length of cycle in days}} \cdot \text{Using this formula, the daily retentions needed by the 6 subjects to cover menstrual losses were 0.18, 0.22, 0.35, 0.56, 0.73 and 1.49 mg (table 4). For replacement of menstrual losses, subject F needed to retain 8 times the amount of iron needed by subject A.}$$

### *Growth*

To find the retention required for growth, the estimated values of Heath and Patek ('37) for the yearly retention of iron needed by girls were used. For 5 subjects the value 145 mg, for girls between the ages of 13 to 14, was used; for subject A the value for the next age group, 170 mg, was used because she had passed her 14th birthday. The values were divided by 365 to convert them into the daily retentions required; these were 0.40 and 0.46 mg per day. Obviously these are average values and girls who are growing faster or slower than the average need more or less. According to Heath and Patek ('37) these values were based on estimates of annual gains of circulating iron and the non-available tissue iron (extra-circulating iron) which increases with growth. The annual gains of circulating iron were estimated on the basis

of the gain in total circulating hemoglobin. This in turn was based on the average hemoglobin in total blood volume for different ages. The annual gain in extra-circulating or unavailable tissue iron was calculated from the arbitrary standard of 5 mg of iron per kilogram of body weight. The authors state that their data are only approximate but that they supply specific information which will be helpful in reaching logical conclusions.

### *Adequacy of the dietary intake*

The adequacy of the two levels of dietary intake can be assessed by comparing the retentions observed with the retentions needed by the subjects. By adding together the amounts needed for replacement of menstrual losses and the

TABLE 5

*Estimated retentions required and observed retentions on two levels of iron intake*

SUBJECT	ESTIMATED RETENTION REQUIRED <sup>1</sup>	OBSERVED RETENTIONS	
		Average intake 8.6 mg	Average intake 11.7 mg
	<i>mg/day</i>	<i>mg/day</i>	<i>mg/day</i>
A	0.64	0.80	1.15
B	0.62	1.54	0.33
C	0.75	1.23	1.54
D	0.96	1.22	2.22
E	1.13	1.42	1.76
F	1.89	0.86	2.10
Average	1.00	1.18	1.52

<sup>1</sup> The estimated retention required is the amount of iron needed for growth and the amount needed for replacement of menstrual losses.

amounts estimated as being required for growth, the following are the minimum retentions required by the 6 subjects: 0.62, 0.64, 0.75, 0.96, 1.13 and 1.89 mg per day (table 5). Wide differences in the retentions required are apparent. They are due mainly to differences in menstrual losses. Three subjects had menstrual losses which were very small, so small that the amount of iron needed to replace these losses was less than the amount estimated as needed for growth.

When the values for the 6 subjects are averaged, the daily retention required is 1.00 mg and the observed daily retentions from the two diets are 1.18 and 1.52 mg (table 5). Thus the average retentions observed on the two diets were greater than those required. From these averages one might conclude that the intake of 8.6 mg of iron per day was adequate for these girls. The average should not be used alone, however, in drawing a conclusion. Since only 6 cases make up these averages, the performance of the individuals should be examined more closely. The average intake of the first period, 8.6 mg per day, was not sufficient to cover the needs of subject F. Her menstrual losses were larger than those of the others, hence her needs were larger. While her losses were large, comparison of them with those of women reported in the literature leads to the belief that such losses are not uncommon. For the other 5 subjects, this dietary intake was possibly sufficient if the criterion for adequacy is merely that the retentions observed are larger than the individual estimated required retentions (table 5). The size of the margin between the observed retentions and those required, however, should also be examined. This margin should be large enough to take care of any small additional needs such as those for replacement of iron lost in sloughed-off skin cells and hair, or those which might be due to an accelerated growth rate. The margin observed for three subjects on this dietary intake was small.

The diet containing an average of 11.7 mg of iron daily was adequate for these girls with the exception of subject B, whose retention appeared to be atypical. For 4 subjects, a wide margin of safety was observed which should easily take care of any small additional needs for iron. During this period subject F, who exhibited the largest menstrual losses, retained enough to cover her total needs and to have a margin of safety. Her actual intake was 13.3 mg per day, which was above the average for the group.

Any recommendation for a dietary intake of iron based on only 6 cases should be a generous one. The possibility should be recognized that the retentions found on the intakes used

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# THE GROWTH-PROMOTING EFFECT ON THE RAT OF SUMMER BUTTER AND OTHER FATS

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While the present authors were carrying out experiments on rats fed a fat-free diet for the purpose of establishing the existence or non-existence of fat soluble factors other than those already known, the work of Boer et al. ('47a, b) on vaccenic acid as a growth-promoting factor was published. As we at that time had been unable to detect the presence of any heretofore unrecognized fat soluble factors essential for growth in the rat, we decided to determine to what extent the findings of Boer et al. would influence our further work.

The presence in butter of unidentified nutritional factors not present in vegetable fats has received the attention of many investigators in recent years. Boutwell et al. ('41, '43) concluded that the superior growth-promoting properties of butterfat as compared to certain vegetable oils is probably due to a saturated compound present in butterfat in small amounts. In a subsequent paper these authors found that the superior growth of rats fed butterfat as compared to that of rats fed corn oil was manifest only when the sole carbohydrate in the diet was lactose. Brown and Bloor ('45) investigated the nutritive value of fractions of fatty acids of butter and whole butterfat and found that the efficiency of a whole butterfat diet in producing gains in body weight of rats was either matched or improved upon by the liquid fatty acid fractions. Henderson et al. ('45) and Jack et al. ('45), in their nutritional studies on milk fat, found that a

diet containing milk fat had no advantage in so far as rat growth is concerned over one containing triolein prepared from olive oil.

Boer ('41) concluded that butterfat contains a new factor necessary for normal growth in rats. Later Boer and Jansen ('42) confirmed the presence in butter of a substance needed for normal growth in rats which is absent from, or present in smaller amounts in, margarine, peanut oil, olive oil, and other fats. The same group (Boer et al., '46) also reported that summer butter, which they define as butter from cows on green pasture, contains a fat soluble substance different from all known vitamins which promotes growth in rats, and that this factor is found in the fatty acid fraction with more than 14 carbon atoms. In more recent work (Boer et al., '47a, b) this factor was finally identified as vaccenic acid.

Deuel et al. ('44) reported that no difference in the growth of weanling rats was noted at any time over a 12-week period when they were fed a butter diet in contrast with results obtained with diets containing corn, cottonseed, peanut or soybean oils, or margarine. In further work, Deuel et al. ('48)<sup>1</sup> found that no difference in the rate of growth of male and female rats was noted over a 6-week period when the diet contained butterfat or cottonseed oil and, further, that no stimulating effect on growth was produced by the administration of vaccenic acid or partially hydrogenated china-wood oil to rats on a rapeseed oil diet. They concluded that vaccenic acid plays no specific role in the growth of the rat.

Recently, von Euler and von Euler ('48)<sup>2</sup> have confirmed the findings of Deuel et al. by observing the growth of young rats through a 25-day test period. In earlier work von Euler et al. ('43) found no evidence of the presence of any special nutritive factors in butterfat which were not also present in margarine. Nath et al. ('48a)<sup>3</sup> separated September butterfat into a liquid and a solid fraction by low temperature sol-

<sup>1</sup>Published since completion of the present experiments.

<sup>2</sup>See footnote 1.

<sup>3</sup>See footnote 1.

vent fractionation. The liquid fraction gave a growth rate in weanling rats superior to that produced by whole butterfat or corn oil, while the solid fraction supported a significantly slower growth rate. In later work ('48b)<sup>4</sup> this group studied the alleged growth-promoting property of vaccenic acid and concluded that this fatty acid does not have any growth-promoting effects on rats fed diets containing corn oil or olive oil.

To clarify the apparent conflict which exists between the results of Deuel et al. and other authors on the one hand, and those of Boer et al. on the other, as well as to get a further insight into the requirements of the rat for as yet unidentified fat soluble growth factors, the following experiments were undertaken.

#### EXPERIMENTAL

The rats used in these experiments were male albinos of the Sprague-Dawley strain. At weaning they were divided into groups of 10 each balanced as to weight, placed in individual wire-floored cages and given the diets shown in table 1 and distilled water ad libitum. The fat soluble vitamin supplements were administered three times weekly in small glass supplementing dishes. Individual weights of the rats were obtained at the start of the experiment and once weekly for a feeding period of 70 days. The feeds and the vitamin supplements were stored until used in a refrigerator under nitrogen. At the end of the experiment the rats were killed, eviscerated, their body length determined (from nose to base of tail), and ground in a meat grinder. Composite samples from each group were analyzed for fat by a cold ether extraction of the finely comminuted carcasses in the presence of an excess of anhydrous sodium sulfate.

The ethyl linoleate used in these experiments was prepared from cottonseed oil by the bromination method of McCutcheon ('42) and stored under a nitrogen atmosphere in a refrigerator until used. The casein was prepared by an exhaustive

<sup>4</sup> See footnote 1, page 84.

TABLE 1  
*Experimental diets fed to different groups of rats*

INGREDIENT	GROUP				
	1	2	3	4	5
			% in diet		
Fat free basal diet <sup>1</sup>	80.0	80.0	80.0	80.0	90.0
Vitamin B complex supplement <sup>2</sup>	10.0	10.0	10.0	10.0	10.0
Summer butterfat	10.0		..	..	.
Oleomargarine fat (Nucoa)		10.0	.	.	.
Olive oil		..	10.0		
Cottonseed oil	..	..	..	10.0	
<i>Composition of fat soluble supplement</i>	<i>Daily dose per rat</i>				
Ethyl linoleate	0.2 ml	0.2 ml	0.2 ml		0.2 ml
Fat soluble vitamins	3	3	3	3	3

<sup>1</sup> The fat free basal diet had the following composition: Ether extracted vitamin test casein, General Biochemicals, Inc., 30.0%; Sure's salts no. 1 ('41) 4.5%; cystine, C.P., 0.3%; sucrose, 65.2%; 2 methyl, 1-4 naphthoquinone, 1 µg per gram of diet.

<sup>2</sup> The vitamin B complex supplement had the following composition: Skellysolve F extracted rice bran concentrate, National Oil Products Co, 966 gm; riboflavin, 0.10 gm; calcium pantothenate, 0.25 gm; inositol, 3.50 gm; choline chloride, 5.00 gm; distilled water to make 1,000 ml.

<sup>3</sup> For groups 1, 2, 3, and 5 the following daily amounts of fat soluble vitamins were fed, dissolved in the ethyl linoleate: crystalline vitamin D<sub>3</sub>, 25 IU, crystalline beta carotene, 50 µg; alpha-tocopherol, 10 mg. In the case of group 4, the same amounts of the vitamins were fed, dissolved in a daily dose of 0.2 ml of cottonseed oil.

ether extraction of vitamin test casein <sup>5</sup> which, when received, had already been extracted repeatedly with ethyl alcohol during its manufacture. The vitamin B complex supplement was prepared from rice bran concentrate <sup>6</sup> by diluting it with distilled water in the ratio of one to three and liquid-extracting this mixture with petroleum ether (Skellysolve F) until a fat free extract was obtained as revealed by the turbidity test of Bloor ('14).

<sup>5</sup> Obtained from General Biochemicals, Inc

<sup>6</sup> Obtained from National Oil Products Co, Harrison, N. J.

## DISCUSSION AND RESULTS

One of the main difficulties encountered in obtaining reliable results is the need to produce a fat free basal diet, particularly when growth-promoting substances that may be active in minute amounts are involved. Mackenzie et al. ('39) showed the difficulties involved in obtaining fat free diets by solvent extraction of yeast and casein. Commercial sucrose was shown by these workers to be a fat free source of carbohydrate. In order to eliminate the possibility of fat contamination by yeast (commonly used as a B complex supplement in low fat diets) and the carbohydrate-containing fraction of the diet, we decided to use fat-extracted rice bran concentrate as the source of vitamin B complex factors and commercial sucrose as the main carbohydrate.

The basal diet, when tested on a group of rats in earlier experiments, produced the typical essential fatty acid deficiency symptoms described by Martin ('39) within 90 days. Since the casein in our basal diet is the only ingredient which, according to our views, may still contain small amounts of fat, it would seem reasonable to assume that an essentially fat free diet could be compounded by replacing casein with a protein hydrolysate which can be made absolutely fat free. This would of course pose the additional problem of producing a hydrolysate that supports normal growth. This problem is now being further investigated in this laboratory.

The results given in table 2 show that there are no heretofore unrecognized fat soluble growth-promoting factors for the rat (vaccenic acid or others) present in summer butter-fat which are not also present in margarine fat<sup>7</sup> and cottonseed oil to the same extent as measured by growth response. This conclusion is supported by comparison of weight gains as well as mean body lengths of the rats. That the formation of adipose tissue in the carcasses of the rats does not influence these conclusions is seen by a comparison of the carcass fat content determined for each group. Group 4, which was

<sup>7</sup> Nucoa.

fed cottonseed oil, showed the largest gain both as to weight and body length, but a statistical evaluation<sup>8</sup> of the data proved the difference to be non-significant. Cottonseed oil, however, gave a significantly greater growth response than did olive oil. Group 5, which received no other fat than ethyl linoleate, showed a significantly lower response both as to growth and body length than the groups that were fed

TABLE 2  
*Results of feeding experiment*

CATEGORY OF INTEREST	GROUP				
	1	2	3	4	5
Mean daily weight gain per rat in grams from 30 through 100 days of age	4.28	4.27	3.99	4.42	3.63
Standard error of the mean	0.141	0.104	0.0950	0.124	0.0890
Average daily food intake in grams per rat	17.2	17.3	16.4	19.7	20.2
Mean body length in millimeters at 100 days	243.0	245.0	241.4	246.7	235.9
Standard error of the mean	1.36	1.14	1.31	1.78	1.74
Per cent fat in eviscerated carcasses	14.91	14.56	15.89	14.26	12.47

summer butterfat, margarine fat, and cottonseed oil. This would seem to indicate that all the fats fed in these experiments stimulated the growth of rats when added to a fat free diet. These results are in accord with those of Deuel et al. ('47).

Our conclusions with reference to vaccenic acid are in contradiction to the results of Boer et al. ('47a, b), who claim

<sup>8</sup>Our criterion for significant difference was as follows:  $\frac{m_1 - m_2}{\sqrt{E_1^2 + E_2^2}}$ , where  $m_1$  and  $m_2$  are the two mean results and  $E_1$  and  $E_2$  are their respective standard errors. If the figure derived from this expression was two or more, the difference was considered significant.

that summer butterfat contains a rat growth-promoting substance, later identified by them as vaccenic acid, present in smaller amounts in, or absent from, certain vegetable oils. In their work on the vaccenic acid content of various fats and oils Geyer et al. ('47) have shown cottonseed oil to contain no vaccenic acid. Therefore, since in our work the group of rats fed cottonseed oil showed the greatest gain in weight and body length, we may conclude that vaccenic acid is of no significance as a growth-promoting factor. These conclusions are in accord with those of Deuel et al. ('48), von Euler and von Euler ('48), and Nath et al. ('48b).

#### SUMMARY

Rat growth experiments were conducted over a 70-day period in which summer butterfat, margarine fat, cottonseed oil, and olive oil were added at levels of 10% to an otherwise fat free basal ration capable by itself of producing essential fatty acid deficiency symptoms. Using growth and body length measurements as criteria, it is concluded that there are no heretofore unrecognized fat soluble growth factors such as vaccenic acid present in summer butterfat which are not also present to the same extent in margarine fat<sup>9</sup> and cottonseed oil. The addition of each of the above fats to a fat free diet produced a significant growth stimulation.

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<sup>9</sup> See footnote 7, page 87.

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# INTESTINAL SYNTHESIS OF NIACIN AND THE METABOLIC INTERRELATIONSHIP OF TRYPTOPHAN AND NIACIN IN THE RABBIT

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TWO FIGURES

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Ample evidence has been presented showing that several species have the ability to synthesize niacin from tryptophan. Krehl et al. ('45) found that the addition of corn to a low protein diet produced a niacin deficiency in the rat, a species which had not been shown before to require this vitamin. The animals grew very poorly, and normal growth could be restored by the addition of either niacin or tryptophan to the diet. Woolley ('45) has shown that niacin deficiency in the mouse produced by the feeding of acetylpyridine, an anti-vitamin for niacin, can be cured by the addition of tryptophan. Niacin deficiency has also been produced in the chick; in this species the criteria of deficiency have been poor growth, poor feathering and perosis. These symptoms respond to tryptophan therapy (Briggs, '45). Interchangeability of niacin and tryptophan has been reported for the dog (Hundley, '47; Singal et al., '48), cotton rats (Schweigert and Pearson, '48) and pigs (Luecke et al., '47).

On the other hand, it has been demonstrated that, as in the case of other water soluble vitamins, niacin is not essential for growth in the ruminants (Pearson, Schmidt and Mackey,

'39; McElroy and Goss, '39; Winegar, Pearson and Schmidt, '40; Lardinois et al., '44; and Johnson et al., '47). Apparently in this case the bacterial synthesis of niacin in the rumen of these species is large enough to meet their requirements.

The effect of niacin deficient diets on the growth of rabbits has been reported by Wooley and Sebrell ('45). These investigators found that the growth of rabbits was depressed by a niacin deficiency when they received a diet which contained 20% casein. There was no difference in the niacin content of the liver, kidney or muscle in rabbits receiving diets deficient in and supplemented with this vitamin. In a later study Wooley ('47) reported that supplementation with either niacin or tryptophan for 8 weeks produced an increase of niacin in the liver of rabbits which had been previously fed a diet low in this vitamin. There was no significant difference in the niacin content of the muscles of the group fed the tryptophan supplemented diet as compared with those of the group fed the deficient diet. The report of Wooley and Sebrell ('45) that niacin is required for normal growth in the rabbit is surprising, especially because of the work of Hundley ('47) and Schweigert and Pearson ('48), who found little or no response when niacin was fed to rats receiving diets containing 20% casein. Furthermore, rabbits synthesize large amounts of riboflavin, pantothenic acid, biotin and folic acid (Olcese, Pearson and Schweigert, '48), which probably are utilized by the animal in the course of the function of "reintestination" or "physiological coprophagy." This peculiarity of the rabbit makes this species similar to the ruminants as far as its requirement for the B vitamins is concerned, and a substantial synthesis and utilization of niacin would be expected in the rabbit. Since the results secured by Wooley did not seem to support this viewpoint, it was important to reinvestigate the need of the rabbit for niacin and to obtain more data on the niacin-tryptophan interrelationship in this species. Balance studies have been conducted in the course of this work to determine the extent of intestinal synthesis of this vitamin in the rabbit. Rate of growth and niacin and N'-methylnico-

tinamide excretion in the urine are the criteria used in interpreting the niacin-tryptophan interrelationship.

# EXPERIMENTAL

Two series of experiments were conducted with New Zealand white rabbits 8 weeks old and with weights ranging from about 800 to 1,200 gm in the first experiment and from 1,000 to 1,600 gm in the second. The rabbits were housed in a room which was equipped with a heater and air cooling units. The room temperature was maintained at about 75°F. to 80°F. at all times. The rabbits were kept in groups in cages provided with wire mesh floors; water and food were given ad libitum.

TABLE 1  
*Composition of the diets*<sup>1</sup>

INGREDIENTS	DIET NO.		
	1 and 2	3 and 4	5 and 6
	<i>gm</i>	<i>gm</i>	<i>gm</i>
Purified casein	20.0	20.0	30.0
Cerelose	58.5	58.28	48.5
Wood pulp	10.0	10.0	10.0
Tryptophan		0.22	
Salts IV <sup>2</sup>	3.0	3.0	3.0
A and D oil	0.5	0.5	0.5
Corn oil	8.0	8.0	8.0

<sup>1</sup> The diets were supplemented with the following vitamins in milligrams per kilogram: mixed tocopherols 500, choline chloride 2,000, inositol 100, 2-methyl, 1-4 naphthoquinone 0.75, pyridoxine 7, thiamine 7, riboflavin 7 and calcium pantothenate 10. Diets 2, 4 and 6 were supplemented with 600 mg of niacin per kilogram.

<sup>2</sup> D. M. Hegsted, R. C. Mills, C. A. Elvehjem and E. B. Hart. *J. Biol. Chem.*, 138: 459, 1941.

The rabbits were distributed so as to have the same number of males and females in the different groups. Otherwise the groups were formed by random selection. The composition of the diets used is shown in table 1. During the first few days of the experiment the purified diets were mixed with the stock diet to make them more acceptable to the rabbits. The addition of tryptophan to rations 3 and 4 containing

20% casein was done so as to bring the total tryptophan content of these rations to the same level as that of rations 5 and 6, which contained 30% casein.

For the metabolism studies individual metabolic cages with double bottom wire floors were used. The rabbits were placed in these cages after the 4th week on the experimental diets. In both experiments collections were made from the different groups at the same time in order to obtain data for comparable periods for all groups. The rabbits were placed in the cages for the collection of the urine and feces for a period of 72 hours. The feed consumption for the three-day period was recorded. The urine was collected in bottles containing acetic acid, and at the end of the third day the contents were mixed and the volume made up to 1,000 ml with water. Appropriate aliquots were neutralized and used for assay. The feces were dried for 24 hours at 60°C., weighed, and finely ground. Samples were then taken for determination of niacin and N'-methylnicotinamide. Niacin was determined by the microbiological method of Krehl, Strong and Elvehjem ('43) using *L. arabinosus* as the test organism and after the niacin was liberated by autoclaving the samples with  $H_2SO_4$  for 30 min. N'-methylnicotinamide was determined in the urine by the fluorometric method of Huff, Perlzweig and Tilden ('45) without any treatment of the samples previous to the assay.

#### RESULTS AND DISCUSSION

The first experiment was designed to test whether rabbits fed purified diets with and without niacin would synthesize this vitamin. Ten rabbits were divided into two groups of 5 each. One group was fed diet 2, which was supplemented with 60 mg of niacin per 100 gm of diet. The other group was fed diet 1, which was not supplemented with niacin. Analysis showed that it contained 0.3  $\mu g$  of niacin per gram. The rabbits were maintained on these diets for 12 weeks. During this time the rabbits in both groups made good gains; the growth data on this experiment are shown in figure 1. The rabbits fed the diet supplemented with niacin made the best

gains, with an average gain for the 12-week period of 1,637 gm. In the group fed the niacin deficient diet one rabbit died during the second week of the experiment. He had made satisfactory gains and his death is not attributed to a niacin deficiency. The other 4 rabbits in this group made an average gain of 1,057 gm in the 12-week experimental period.

The difference in the growth of the two groups of rabbits is statistically significant, showing that depression in growth resulted from the deficiency of niacin. This depression

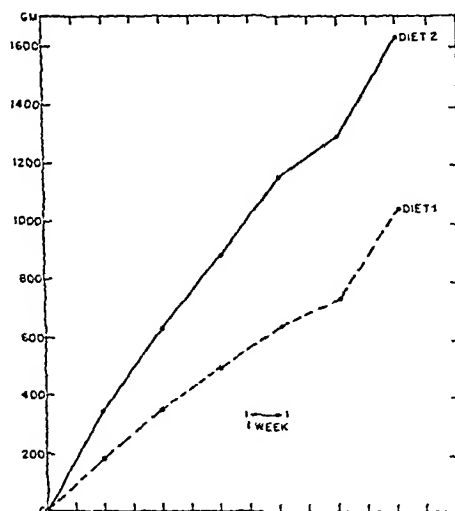


Fig. 1 Effect of niacin supplementation on the growth of rabbits. Diet 1, basal with no niacin; diet 2, basal plus niacin.

of growth is probably related to food consumption. Wooley and Sebrell ('45) found, in the course of studies with purified diets similar to the ones used in this experiment, that anorexia was the only symptom of niacin deficiency in the rabbit. In the present studies careful observations made on the rabbits fed the deficient diet failed to show any of the characteristic symptoms of niacin deficiency that have been reported for other species. Neither blacktongue nor diarrhea was present at any time. Dermatitis did not appear either, but a strong tendency of the rabbits to eat each other's hair was shown

by rabbits in both groups. This phenomenon, which had been observed before in the course of studies on B vitamin synthesis in the rabbit, is not attributed to a niacin deficiency since the symptom was shown by rabbits in both groups.

At the end of the 4th week the rabbits were started on metabolism studies. Urine and feces were collected for two different periods for each rabbit. Niacin was determined in the feed, feces and urine and N'-methylnicotinamide in the urine. A total of 8 collections was made of the urine and feces of rabbits from the niacin deficient diets and 10 collections were made of the urine and feces of rabbits receiving

TABLE 2  
*Ingestion and excretion of nicotinic acid by rabbits fed low and high  
nicotinic acid diets*  
(Values expressed in micrograms per 24 hours)

	LOW-NIACIN GROUP	NIACIN-SUPPLEMENTED GROUP
Niacin ingested	11.2	25280.0
Niacin excreted in:		
urine	139.9 (8) <sup>1</sup>	3059.7 (10)
feces	38.8 (8)	64.1 (10)
total	178.7	3123.8
N'-methylnicotinamide in urine	137.8 (8)	290.0 (10)

<sup>1</sup> The figure in parentheses gives the number of collection periods.

the supplemented diet. The data on the excretion of niacin and N'-methylnicotinamide are shown in table 2. In the group fed the niacin deficient diet the average daily ingestion of niacin was 11.2  $\mu$ g; the combined excretion in the urine and the feces was 178.7  $\mu$ g of niacin per day. This 16-fold increase in the amount of niacin excreted over the amount ingested is very significant and definitely shows that substantial synthesis occurred. The fact that the fecal excretion of 38.8  $\mu$ g was higher than the ingestion indicates that niacin was being synthesized in the intestine of the rabbit. This is in agreement with similar results found with this species in the case



of other B vitamins (Olcese et al., '48). The values for N'-methylnicotinamide excretion were consistently higher in the rabbits which received niacin. These animals excreted an average of 290  $\mu$ g of N'-methylnicotinamide daily, as compared with 138  $\mu$ g for the rabbits on the niacin deficient diet. The significance of the difference in the excretion of N'-methylnicotinamide is questionable, since in the subsequent series the values for the niacin deficient and supplemented rabbits were nearly the same.

### *Effect of level of tryptophan intake*

This series was designed to obtain data on the conversion of tryptophan to niacin and on the growth response of rabbits fed various combinations of these supplements. Six groups of 5 rabbits each were used. They were started on diets 1, 2, 3, 4, 5 and 6. The first 4 diets contained 20% casein and were devised primarily to test the response of the rabbit to niacin and tryptophan. Rations 5 and 6 contained 30% casein and were used for the purpose of determining if tryptophan fed as casein is as effective in preventing niacin deficiency as when fed in the form of free amino acid.

The average growth of the rabbits in the different groups in this experiment is shown in figure 2. Some rabbits in the different groups refused to eat the purified diets and there was some difficulty in getting them started on the diets. The rabbits which received diet 1 showed a particular aversion for the purified diet. This accounts for the sharp decline in growth shown by these rabbits (fig. 2) at the beginning of the experiment. Since rabbits practice functional coprophagy, the question is raised of the possibility that under conditions of natural diets, which are more palatable than the purified diets, the problem of a niacin deficiency might not be so serious. It should be pointed out that the only symptom attributed by Wooley and Sebrell ('45) to niacin deficiency in rabbits fed purified diets was anorexia. It is interesting to note that these investigators found rabbits that made gains when fed the niacin deficient diets. In the present experiment there

was also one rabbit fed the deficient diet which made fairly good gains. However, the rabbits on the low niacin intake lost an average of 34 gm in 10 weeks. The rabbits which received ration 2 with 20% casein and niacin gained an average of 680 gm in 10 weeks. The rabbits which received ration 3 with 20% casein and tryptophan gained 864 gm. The rabbits which received ration 4 with 20% casein plus niacin and tryptophan gained 693 gm. The rabbits receiving ration 5 with

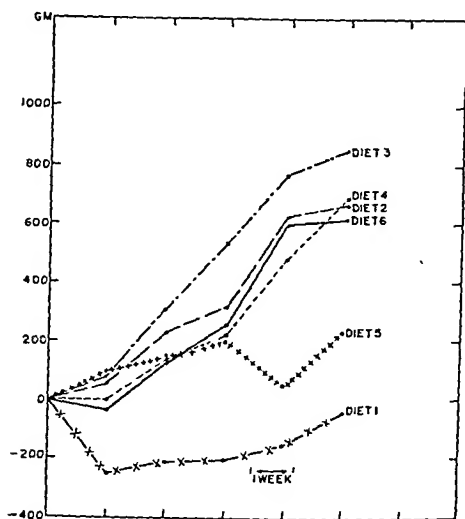


Fig. 2 Effect of different diets on the growth of rabbits. Diet 1, basal with 20% casein and no niacin; diet 2, basal plus niacin; diet 3, basal plus tryptophan; diet 4, basal plus niacin and tryptophan; diet 5, 30% casein without niacin; and diet 6, 30% casein plus niacin.

30% casein but no niacin or tryptophan made an average gain of only 247 gm, while the rabbits receiving ration 6 with 30% casein plus niacin gained 629 gm.

Comparing the growth of the rabbits which received casein without added nicotinic acid with that of those receiving 20% casein plus tryptophan at the same level as the 30% casein group, it can be seen that the gains in weight of the group receiving the free amino acid were much higher than in the case of the rabbits receiving the extra tryptophan from casein.

This seems to support the finding of Rosen et al. ('46) that while tryptophan, administered as the free amino acid, may serve as an excellent source of niacin, tryptophan ingested as protein is considerably less effective.

Several rabbits in the different groups died. One rabbit receiving the niacin deficient diet and one receiving the tryptophan supplemented diet died the first week of the experiment. The three rabbits that refused to eat in the group fed the 20% casein basal diet died from the 7th to the 10th week. During the 8th week one rabbit in the group fed 20% casein plus niacin developed paraplegia and was killed. Two rabbits

TABLE 3  
*Urinary excretion of niacin and N'-methylnicotinamide by rabbits*

DIET NO.	TREATMENT DIETARY	INGESTION OF		EXCRETION IN URINE	
		Niacin	Tryptophan	Niacin	N'-methyl-nicotinamide
		$\mu\text{g}/24 \text{ hr}$	$\text{mg}/24 \text{ hr.}$	$\mu\text{g}/24 \text{ hr}$	$\mu\text{g}/24 \text{ hr.}$
1	Basal (20% cas.)	12.1	177.8	75.6	552.3
2	Basal + niacin	24,180.0	177.2	1,911.3	528.0
3	Basal + trypto.	10.3	226.8	176.5	758.9
4	Basal + niacin + trypto.	31,080.0	341.8	2,596.3	663.5
5	30% casein	9.9	218.3	167.3	818.1
6	30% casein + niacin	23,100.0	254.0	1,716.3	710.3

fed 30% casein died during the 5th week of the experiment and one fed 30% casein plus niacin died in the 6th week.

Studies on the urinary excretion of niacin and N'-methylnicotinamide were conducted starting the 6th week. The average values obtained in these studies are shown in table 3. The urinary excretion of niacin by the rabbits in the group receiving the basal diet was 75.6  $\mu\text{g}$  per day. The rabbits which received a diet supplemented with tryptophan and those which received a diet with 30% casein excreted in the urine an average of 176.5 and 167.3  $\mu\text{g}$  of niacin per day, respectively. This 2.5-fold increase in excretion by the rabbits on

the basal diet shows that an increased synthesis of niacin resulted from the feeding of extra tryptophan. The fact that the basal diet contains 0.66% as much tryptophan as diets 3 and 5 indicates that the tryptophan is first used by the rabbit to meet its requirements for this amino acid and that the synthesis of niacin occurs at the maximum rate only after the requirements for tryptophan per se are met. This suggests that the conversion of tryptophan to niacin occurs within the tissues of the animal itself and is not due to increased intestinal synthesis, since if the latter were the case it is difficult to explain how an increase of 33% in the tryptophan in the diet would bring a 2.5-fold increase in the excretion of niacin.

The rabbits fed diets 2, 4 and 6, which were supplemented with niacin, excreted much higher amounts of niacin through the urine than the other animals but still only a small fraction of the total amount ingested.

The values for the urinary excretion of N'-methylnicotinamide in this experiment are shown in table 3. The average daily excretion of N'-methylnicotinamide in the urine of the rabbits fed the low niacin and 20% casein diet was 552.3  $\mu$ g as compared with 528  $\mu$ g for the rabbits fed a diet containing 20% casein plus niacin. There was considerable variation within a group and the differences between these two groups are not considered significant. The results secured in this case are in agreement with the finding of Huff and Perlzweig ('43) that there is no increase in the urinary excretion of N'-methylnicotinamide after the feeding of niacin to the rabbit. The other 4 groups of rabbits receiving additional tryptophan, either as free amino acid or higher levels of casein, excreted in the urine larger amounts of N'-methylnicotinamide than the two previous groups. The rabbits fed 20% casein plus tryptophan excreted 758.9  $\mu$ g of N'-methylnicotinamide, as compared with 663.5  $\mu$ g by the rabbits fed 20% casein plus niacin and tryptophan. The rabbits receiving the 30% casein diets without and with casein excreted 818.1  $\mu$ g and 710.3  $\mu$ g of N'-methylnicotinamide, respectively. On the whole no great variations among the groups were found.

The data presented in this paper show that niacin is synthesized by the rabbit by both microbial activity in the digestive tract and metabolic processes within the tissues. The fact that tryptophan fed as the free amino acid gave a better growth response than when fed in the form of casein may be taken as an indication that free tryptophan is superior for synthesis of nicotinic acid than that found in protein. However, it may also be that the increased rate of protein metabolism increases the demand for niacin. The administration of an unbalanced protein in this case would decrease the efficiency of tryptophan as a source of niacin.

# SUMMARY

1. The feeding of a niacin deficient diet to rabbits resulted in a decreased gain in weight. One group of rabbits fed a purified diet supplemented with 60 mg of niacin per 100 gm of diet showed an average gain in weight of 1,637 gm, as compared to a gain of 1,057 gm made by a group that received a similar diet without the addition of niacin.

2. Balance studies showed that considerable amounts of niacin were being synthesized by the rabbits fed the low niacin diet. The average daily ingestion of niacin for this group was 11.2  $\mu$ g, with a corresponding niacin excretion of 178.7  $\mu$ g. The average daily fecal excretion of niacin amounted to 38.8  $\mu$ g, showing that synthesis was taking place in the digestive tract.

3. Growth data secured with 6 groups of rabbits fed different levels of niacin and fed tryptophan as the free amino acid and as additional protein showed that niacin could be synthesized from its precursor tryptophan. One group of rabbits receiving the basal diet with 20% casein but no additional tryptophan or niacin lost an average of 34 gm in 10 weeks. The group receiving the basal diet supplemented with niacin gained an average of 680 gm in the same period. Rabbits receiving the basal ration plus tryptophan gained 864 gm, and the rabbits in the control group receiving the basal

diet supplemented with both niacin and tryptophan gained 693 gm.

4. Tryptophan fed as protein was not as effective as when fed as free amino acid, as evidenced by the fact that the rabbits in a group fed additional tryptophan in the form of casein gained only 247 gm as compared to the 864 gm gained by the group which was fed the same amount of tryptophan in the form of free amino acid.

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# URINARY PANTOTHENATE, BLOOD GLUCOSE, AND INORGANIC SERUM PHOSPHATE IN PATIENTS WITH METABOLIC DISORDERS TREATED WITH DOSES OF PANTOTHENATE<sup>1</sup>

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## INTRODUCTION

Evidence has accumulated that pantothenic acid participates at some stage in the metabolism of carbohydrate. Pilgrim et al. ('42) and Pilgrim and Elvehjem ('44) found a decreased rate of oxygen consumption in the presence of pyruvate by liver homogenates of pantothenic acid deficient rats. Recent investigations have shown that coenzyme A, which is involved in the utilization of acetate and possibly in the breakdown of pyruvate to acetate, contains large amounts of pantothenic acid (Lipmann et al., '47; Novelli and Lipmann, '47; Kaplan and Lipmann, '48). Animals on a pantothenic acid deficient diet for two to three weeks show a gradual depletion of coenzyme A in their tissues (Olson and Kaplan, '48), an inability to acetylate injected *p*-aminobenzoic acid (PABA) (Riggs and Hegsted, '48), and adrenal cortical damage if the diet is continued long enough (Daft and Sebrell, '39). In spite of the many reports on the role of

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pantothenic acid in animals, its role in human subjects has not as yet been demonstrated.

This study is concerned with an attempt to evaluate the role of pantothenic acid in patients with diseases of metabolism involving the carbohydrate cycle. The patients studied had either diabetes, Addison's disease, or cirrhosis of the liver and, in addition, two patients with chromophobic tumor of the pituitary were studied. Amounts of pantothenate far above the presumed daily requirement were given to the subjects in the form of either calcium pantothenate or panthenol. The daily dose was from 12 to 20 gm, one-third at each meal.

#### EXPERIMENTAL

Whole blood was collected in vials containing oxalate and precipitated immediately. Blood sugar was determined on the filtrate by the Nelson modification of the Somogyi method (Nelson, '45). Inorganic phosphate was determined on serum by the Fiske and Subbarow ('25) method modified for the Coleman colorimeter. Pantothenic acid was determined in urine by the methods of Atkin et al. ('44) and Skeggs and Wright ('44).

The first observations were concerned with the excretion of pantothenic acid by the subjects. Urine samples were collected for 24 hours while the patients were on their regular diets. The diets were then supplemented with 12 to 20 gm of pantothenate daily as indicated. On normal diets the excretion of pantothenic acid ranges from 2.1 mg to 5.0 mg, with an average of 3.7 mg (Rubin et al., '48; Sarett, '45). The 24-hour excretion of pantothenic acid by the subjects in this study is shown in table 1. The range was from 2.6 mg to 8.6 mg per 24 hours. The latter figure, in a patient with cirrhosis of the liver, may reflect the inclusion in the diet of vitamin supplements of which we had no knowledge. The administration of panthenol, in equivalent doses of calcium pantothenate, was associated with a greater urinary excretion of pantothenate, as is shown in the table. The same results have been reported by Rubin et al. ('48).

TABLE 1  
*The 24-hour excretion of pantothenate*

CASE	DIAGNOSIS	DIET	DAILY DOSE CALCIUM PANTOTHENATE OR "PANTHENOL"	CA. PAN. EXCRETED	% DOSE EX- CRETED	REMARKS
				<i>mg/24 hr.</i>		
MA	Addison's disease	Hospital	None	4.7		Average of 3 urine samples 24 hr. each
MA	Addison's disease	Hospital	20 gm Ca Pan.	2,900.0	14.4	Average of 3 urine samples 24 hr. each
MA	Addison's disease	Hospital	17 gm Panthenol	12,500.0	62.4	Average of 5 urine samples 24 hr. each
GA	Addison's disease	Hospital	None	4.0		
GA	Addison's disease	Hospital	12 gm Ca Pan.	2,100.0	17.5	Calculated on basis of mg/ml; 24-hr. urine samples not available
HI	Chromophobic tumor	Hospital	None	6.0		
NE	of pituitary	Hospital	15 gm Ca Pan.	1,400.0	9.0	Average of 4 urine samples 24 hr. each
LO	Cirrhosis of liver	Hospital	None	6.9		Average of 3 urine samples 24 hr. each
GR	Cirrhosis of liver	Hospital	None	2.6		
GR	Cirrhosis of liver	Hospital	15 gm Panthenol	2,800.0		12-hr. collection (overnight)
GR	Cirrhosis of liver	Hospital	15 gm Panthenol	4,400.0		12-hr. collection (overnight)
VA	Cirrhosis of liver	Hospital	None	8.6		Average of 2 urine samples 24 hr. each
VO	Diabetes mellitus	Home diet	None	4.2		On 8 units of insulin daily

TABLE 2

*Excretion of urinary pantothenate after glucose or insulin administered intravenously*

CASE	DIAGNOSIS	THERAPY	URINARY EXCRETION OF CALCIUM PANTOTHENATE					
			Before glucose			After glucose		
			Urine Vol.	Ca Pan.	mg/ml	Urine Vol.	Ca Pan.	mg/ml
GA	Addison's disease	None	575	0.0026	150	0.0033		
GA	Addison's disease	Ca Pan. 12,000 mg/day	178	0.1400	210	0.1400		
MA	Addison's disease	"Cortin" and Doca <sup>1</sup>	2000 <sup>2</sup>	0.0020	310	0.0020		
MA	Addison's disease	Doca <sup>1</sup>	1630 <sup>2</sup>	0.0030	288	0.0030		
MA	Addison's disease	Ca Pan. 15,000 mg/day	800	0.8800	365	0.6000		
NE	Pituitary tumor	None	80	0.0130	190	0.0140		
HI	Pituitary tumor	None	1080	0.0060	246	0.0040		
AU	Cirrhosis of liver	"Liver extract" <sup>3</sup>	88	0.0110	246	0.0070		
HO	Cirrhosis of liver	"Liver extract" <sup>3</sup>	5	0.0400	27	0.0300		
LO	Cirrhosis of liver	"Liver extract" <sup>3</sup>	665	0.0040	120	0.0070		
DA	Diabetes mellitus	Diet and insulin	190	0.0090	246	0.0060		
VO	Diabetes mellitus	Diet and insulin	Before insulin 920	0.0047	150	0.0077	After insulin 16	Units insulin I.V.
OR	Diabetes mellitus	Diet	40	0.0047	115	0.0046	20	

<sup>1</sup> Desoxycorticosterone acetate.<sup>2</sup> Twenty-four-hour urines collected up until beginning of test.<sup>3</sup> This liver extract contained 191 µg/ml of pantothenic acid.

In the second part of the study the effect of the intravenous administration of glucose or insulin on the urinary excretion of pantothenic acid was observed. All the tests were done on patients fasted overnight. Pantothenic acid was measured in the urine voided just before the infusion and in the urine collected during three to 4 hours after the injection. Occasionally an overnight or 12-hour specimen was used for the

TABLE 3

*Blood glucose and phosphate changes during intravenous glucose tolerance test in two patients with Addison's disease, before and after administration of pantothenate*

	HOURS AFTER GLUCOSE	BEFORE PANTOTHENATE				AFTER PANTOTHENATE			
		Blood glucose		Inorganic PO <sub>4</sub> (Serum)		Blood glucose		Inorganic PO <sub>4</sub> (Serum)	
		mg %		mg %		mg %		mg %	
CASE MA, MALE	Fasting	80 <sup>1</sup>	72 <sup>2</sup>	3.22 <sup>1</sup>	3.42 <sup>2</sup>	75 <sup>3</sup>		3.42 <sup>3</sup>	
	$\frac{1}{2}$		210	3.00	3.20	248		3.28	
	1	140	148	3.06	3.12	165		3.37	
	2	61	75	3.08	3.24	85		3.48	
	3	60	50	3.00	3.40	58		3.73	
	4	59	62	3.14	3.52	60		3.80	
CASE GA, FEMALE	Fasting	68 <sup>4</sup>		3.86 <sup>4</sup>		78 <sup>5</sup>	70 <sup>6</sup>	4.00 <sup>5</sup>	4.04 <sup>6</sup>
	$\frac{1}{2}$	176		3.58		254	180	3.72	3.78
	1	126		3.72		159	140	3.54	3.78
	2	81		3.74		94	88	3.63	3.90
	3	72		3.74		74	72	3.67	3.83

<sup>1</sup> Receiving 5 ml cortical extract, 2.5 mg DOCA (desoxycorticosterone acetate) and 15 gm salt daily.

<sup>2</sup> No cortical extract for 13 days; receiving 2.5 mg DOCA and 15 gm salt daily.

<sup>3</sup> Receiving 5 ml cortical extract, DOCA, salt, and calcium pantothenate (15 gm for 26 days) daily.

<sup>4</sup> Receiving 20 ml cortical extract, 5 mg DOCA, and salt daily.

<sup>5</sup> Receiving 20 ml cortical extract, DOCA, and salt daily; and calcium pantothenate, 12 gm daily for 7 days.

<sup>6</sup> No cortical extract for 7 days; 5 mg DOCA and salt daily; calcium pantothenate, 12 gm daily for 20 days.

pre-injection assay. Glucose was administered as a 20% solution infused over a period of 30 minutes, the dose being arranged so that the patient received 0.5 gm per kilogram of body weight. Observations under these conditions were made on the patients with chromophobic tumors of the pituitary,

TABLE 4

*Blood glucose and phosphate levels during intravenous administration of glucose or insulin*  
(Levels were determined before and again after the administration of large doses of calcium pantothenate)

	HOURS AFTER GLUCOSE OR INSULIN	BEFORE PANTOTHENATE		AFTER PANTOTHENATE	
		Blood glucose	Inorganic $\text{PO}_4$ (Serum)	Blood glucose	Inorganic $\text{PO}_4$ (Serum)
GLUCOSE I.V.	Fasting	mg %	mg %	mg %	mg %
		88	2.65	91 <sup>1</sup>	2.78 <sup>1</sup>
	$\frac{1}{2}$	270	2.25	246	2.52
	1	131	2.22	102	2.38
	2	58	2.40	68	2.47
	3	76	2.48	79	2.46
INSULIN I.V.	Fasting	232 <sup>2</sup>	2.72 <sup>2</sup>	213 <sup>3</sup>	3.28 <sup>3</sup>
	$\frac{1}{2}$	97	2.04	143	2.74
	1	70	2.50	97	2.76
	2	103	2.80	85	2.86
	3	125	3.48	93	3.34

<sup>1</sup> Patient had received 12 gm of calcium pantothenate daily for 5 days before test.

<sup>2</sup> Patient received 16 units of insulin intravenously for test.

<sup>3</sup> Test repeated 26 days later with 16 units of insulin intravenously; 12 gm calcium pantothenate daily for 7 days prior to test.

on the patients with Addison's disease, on three patients with diabetes, and on three patients with cirrhosis of the liver. The results of administering glucose intravenously are shown in table 2. The excretion of pantothenate was not influenced by raising the blood sugar level. Similarly, the effect of accelerating carbohydrate metabolism by administering insulin

intravenously had no influence on the excretion of pantothenate, as is also shown in table 2.

Table 3 gives the glucose and inorganic phosphate levels during the administration of glucose in the two patients with Addison's disease. The administration of pantothenic acid for varying periods prior to the glucose tolerance test did not affect the fasting levels of glucose or phosphate or alter the nature of the curves. The same type of experiments were done on the patients with chromophobic tumors of the pituitary and on the diabetic patients. Examples of the results of such experiments are given in table 4. Again pantothenate had no appreciable effect on the levels of glucose or inorganic phosphate following the intravenous administration of glucose, nor was the effect of insulin given intravenously modified by the administration of pantothenate.

#### DISCUSSION

The results indicate that the urinary excretion of pantothenic acid is normal in patients with the diseases of metabolism that were included in the group studied. When calcium pantothenate or panthenol was added to the diet, the excretion of pantothenic acid was increased significantly. These findings are the same as were observed in normal subjects by Rubin et al. ('48, '49), and fit in with the observations of Wright et al. ('46) with respect to the renal clearance of pantothenic acid. These authors showed that at ordinary normal plasma concentrations only a trace of pantothenic acid is excreted in the urine, but that on increasing the plasma concentration of the compound by oral or intravenous administration there is an abrupt rise in the clearance of pantothenic acid.

Wright ('42) showed that when glucose was administered to rabbits the blood level of pantothenic acid was decreased, presumably as a result of increasing carbohydrate utilization. In the observations by the present authors the levels of blood pantothenate were not determined, but in these short-term experiments no significant effect on the excretion of

pantothenic acid was observed as a result of glucose or insulin administration.

The fall in serum inorganic phosphate that occurs after the administration of glucose or insulin is probably due to the formation of an intermediate hexosphosphate (Hartman and Bolliger, '25) or to the diffusion of glucose into the tissues with the formation of a phosphate compound (Levine et al., '49). Our results indicate that the administration of excessive doses of pantothenic acid for periods of 5 to 26 days does not influence carbohydrate metabolism as reflected by the blood glucose or phosphate levels following the administration of either glucose or insulin intravenously. It should be borne in mind that no experiments were done on pantothenic acid deficient diets, but that the experiments were conducted while the patients were on normal diets and when these diets were supplemented with excessive amounts of pantothenic acid.

The administration of very large doses of pantothenic acid did not affect the blood glucose levels in the patients with diabetes nor was there any change in the insulin requirement during the period of pantothenic acid administration. This observation is in agreement with the finding (Irwin and Ralli, '48) that in alloxan diabetic rats neither the addition nor absence of pantothenic acid in the diet had any effect on the level of blood sugar or on the urinary excretion of glucose.

#### SUMMARY

The daily excretion of pantothenic acid was studied in patients with diabetes, Addison's disease, cirrhosis of the liver, and chromophobic tumor of the pituitary. On a normal diet the excretion of pantothenic acid in all subjects varied from 2.6 to 8.6 mg per 24 hours. When calcium pantothenate was added to the diet the daily excretion rose so that at least 9% of the ingested pantothenate was excreted. When pantothenol was the source of pantothenate, the excretion of pantothenic acid was still further increased. Insulin and glucose given intravenously did not affect the excretion of pantothenic



acid in any of the patients studied. The blood levels of glucose and inorganic phosphate, determined following the intravenous administration of glucose or insulin, were not influenced by large daily doses of calcium pantothenate.

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# HYPERVITAMINOSIS A IN THE DOG

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SIX FIGURES

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The production of hypervitaminosis A in the rat, the mouse and the guinea pig, with concurrent bony changes and toxic symptoms, has been reported by numerous workers—Takahashi et al. ('25), Harris and Moore ('28), Collazo, Rubino and Varela ('29), Bomskov and Seemann ('33), Collazo and Rodriguez ('33), Drigalski ('33), Moll et al. ('33), Davies and Moore ('34), Strauss ('34-'35), Weslaw et al. ('38), Cornil, Chevallier and Paillas ('39), Noetzel ('39), Wolbach and Bessey ('42), Moore and Wang ('45), Wolbach ('47). It has been recognized as a result of accidental overdosage in man (Rodahl and Moore, '43; Josephs, '44; and Rothman and Leon, '48). The dog, however, has never been studied from this viewpoint. Corroboration in a new species of the findings relating to hypervitaminosis A, and the convenience of using a larger animal because of the need of further work on the chemical aspects of the problem, have prompted the present investigation.

## EXPERIMENTAL

A litter of 5 greyhound puppies born of thoroughbred greyhounds, laboratory animals of several years' standing,

was selected. These puppies were two months old and in good health when the experiment was begun. They had been with the mother until a week before the start of the experimental period. At this time they were transferred to separate cages and put on a diet of milk, bread, horsemeat, dog chow and occasional vitamin D supplement in the form of cod liver oil. Their weights at this time ranged between 4.5 and 5.5 kg. The distribution between the sexes was three males and two females. One of each sex, both of them representing the heavier dogs of the litter and weighing 5 + kg, was selected for excessive vitamin A administration.

### *Dosage and manner of administration*

Vitamin A in the form of concentrate<sup>1</sup> with a potency of 500,000 I.U. per gram was used. This was fed at a level of 300,000 I.U. per kilogram of body weight and was given daily, Sundays excepted. It was administered by mouth for the first 22 days of the experiment. Since the anticipated result was not obtained after this period, it was thought wiser to discontinue the oral route of administration. The dogs were then fed the concentrate by stomach tube and this was followed by either 4.0 ml of corn oil or of olive oil, in order to rinse the vitamin preparation down the tube. No complications in tube-feeding the dogs were encountered.

Two different samples of concentrate, with similar potencies, were employed. Both of them had been repeatedly used on guinea pigs and when fed at the above-mentioned level were known to give a typical picture of vitamin A excess, with fracture of long bones, after a 12- to 16-day period.

### *Procedures followed during the course of the experiment*

The dogs were weighed daily, Sundays excepted. X-rays to determine bony changes were taken of the experimental animals and the controls at the beginning of the experiment

<sup>1</sup> Purchased from Distillation Products, Inc., Rochester, N. Y.

and at intervals thereafter. The clinical course was carefully followed.

The dogs were bled at frequent intervals and except for some of the earlier bloods, which were studied merely for their cholesterol and phospholipid content, determinations were done on specimens obtained after a 12-hour fast. The dogs were followed from the standpoint of serum cholesterol and serum lipid-phosphorus throughout the period under observation. Serum inorganic phosphorus, serum alkaline phosphatase and serum vitamin A levels were followed from the 45th day on. Studies on blood sugar, non-protein nitrogen, uric acid, creatinine, serum sodium and serum chloride were done during the final stages of the experiment. The bromsulphonphthalein test was done on both experimental animals, using normals as controls, once the syndrome was well established.

Serum vitamin A levels were determined by the Lowry-Bessey method (Lowry and Bessey, '45), checked occasionally by the May method (May et al., '40). An unpublished Fiske and SubbaRow method for lipid-P was employed. Cholesterol was determined by the Lieberman-Burchard (Bloor, Pelkan and Allen, '22; Mirsky and Bruger, '32) colorimetric procedure. The Folin-Malmros (Folin and Malmros, '29) micro-method was used for blood sugars. Non-protein nitrogen, uric acid, creatinine and creatine were measured by the routine Folin procedures (Folin, '34). Bodansky's method (Bodansky, '32) for determining serum alkaline phosphatase and serum inorganic phosphorus was utilized. Serum Ca was determined by the Fiske and Adams method (Fiske and Adams, '31), serum chloride by the Whitehorn method (Whitehorn, '20-'21), and serum sodium by Butler and Tutthill's (Butler and Tutthill, '31) method. Bromsulphonphthalein retention, using a 5% solution and administering 5 mg of dye per kilogram of body weight, was measured at the end of 5-, 15-, and 30-minute periods.

## RESULTS

*Weight changes*

The male controls showed a satisfactory weight gain throughout. During the 67-day period under observation they gained on the average 135 and 148 gm a day, respectively. The female control during the first 56 days gained an average of 93 gm daily. A sudden drop then occurred during the next 6 days due to an attack of diarrhea of unexplained origin, followed by a slower return to normal. Comparing the experimental male and female with their respective male and female controls up to the point where a drop in the weights

TABLE 1  
*Daily weight gain in grams — 53-day period*

	CONTROL		EXPERIMENTAL
	Dog 1	Dog 3	Dog 2
Male	160	168	93
Female	Dog 5		Dog 4
	111		73

of the experimental animals took place (53rd day), the average daily weight gains are as indicated in table 1. From the 53rd day on, both experimental animals showed a precipitous fall in weight, the male losing 23% of his maximum weight in 8 days, the female 15% in 5 days.

*Clinical course*

The dogs seemed well and normally active up to and beyond the point where the decline in weight first began to manifest itself. On the 30th day of the experiment it was first noted that their appetites were failing. Solid food, especially meat, was refused either wholly or in part. Except for this they continued to react normally until two to three days after the rather precipitous fall in weight. From then on, both rapidly declined.

The female animal, which seemed to respond more emphatically and promptly to the excessive vitamin A, refused to stand, yelped when touched, and shivered more or less continuously. She acted as though she were in great discomfort. Twenty-four hours later her buttocks were soiled, she smelled strongly of urine, licked her front paws more or less continuously as does a dog which has sustained an injury to them, and was unable to use her hind legs. No fractures could be detected but there was extreme tenderness over all epiphyses. During the last day of life this animal seemed relatively indifferent to her surroundings. She was sacrificed under intravenous pentobarbital anesthesia on the 58th day of the experiment.

The male dog (no. 2), which was maintained on the regime for a longer period of time and was sacrificed on the 69th day under pentobarbital anesthesia, differed from the female in that he showed the effects of a greater weight loss. Skeletal landmarks seemed unusually prominent. The normal dorsal curvature of the spine was exaggerated. Movements of the shoulders would bring the medial borders of the scapulae into profile, as though there were no force to counteract the pull of such muscles around the shoulder joint as had scapular attachments. Bony protuberances, bony ridges and joints seemed more prominent because of excessive and rapid wasting. Hyperesthesia of the skin and extreme tenderness over all extremities were marked in the last few days of life. Unwillingness to stand was a symptom occurring during the last 48 hours, but at no time were gross fractures detectable. Both in this dog and the female a moderate degree of exophthalmos was noted during the last 7 to 10 days of life, but no other ophthalmological changes were noted in either dog.

### *X-ray findings*

Satisfactory x-ray films of a few bones only were obtained at the conclusion of the experiment. The following description (dog 2) is based on the comparison with x-ray films from control dog 1.

The epiphyseal lines of all the long bones of the experimental dog are markedly narrower than those of control bones. The cortices of the femur, tibia, radius and ulna of the experimental dog are less dense and thinner. The most striking change is shown in the fibula, which is markedly reduced in width and density, almost to complete disappearance a few centimeters be-

low the proximal end, whereas the cartilaginous end is of approximately normal width. The tibia shows marked loss of density of the crest just below the epiphysis of the tuberosity and in this location the outline of the bone is fuzzy in appearance. The distal ends of the ulna are markedly expanded, fuzzy, and, in dog 2, the appearance suggests that of an impacted fracture. The distal end of the radius shows narrowing, as compared to that of the control, and the periosteal borders for 2 cm proximal to the epiphyseal line are irregular and fuzzy in outline.

### *Blood chemistry*

Aside from the changes in cholesterol and lipid-phosphorus values, there was little that was remarkable in the chemical blood picture. The non-protein nitrogen rose terminally to a very moderate degree. There was a terminal increase in blood creatine, probably a reflection of excessive muscle wasting. Dog 4 showed an increase in serum calcium. Phosphatase and inorganic phosphorus values lay within normal limits. A terminal and fairly abrupt rise in serum sodium (153 milliequivalents per liter of serum) was present in dog 4. There was little difference between experimental animals and controls as regards bromsulphonphthalein retention, though a slightly increased retention in the hyper A animals was seen, an observation also noted in guinea pigs (Maddock, '42). Serum vitamin A levels, remarkably high normally in the dog (Clausen et al., '40; Wakerlin and Moss, '43), showed an 8- to 40-fold increase (table 2).

Cholesterol and lipid phosphorus values remained unaltered in both experimental animals until symptoms of acute hypervitaminosis A were well established. The female, three days before her exitus, showed a moderate decline in both of these values. This was followed on the final day by a secondary rise which, however, fell short of the previous normal levels. In the male the drop in both cholesterol and lipid-P was more abrupt, and these values remained at a low level until the animal was sacrificed (table 2).



TABLE 2

## Vitamin A, cholesterol and lipid-P

DAY OF EXPERI- MENT	HYPER A DOGS						CONTROL DOGS					
	Dog 2			Dog 4			Dog 1			Dog 3		
	Vitamin A	Choles- terol	Lipid-P	Vitamin A	Choles- terol	Lipid-P	Vitamin A	Choles- terol	Lipid-P	Vitamin A	Choles- terol	Lipid-P
	I.U. %	mg %	mg %	I.U. %	mg %	mg %	I.U. %	mg %	mg %	I.U. %	mg %	mg %
1												
2		230.0	16.15					245.0				
3												
4												
16		255.0	18.39						15.02		155.0	15.55
25		226.0	18.42					235.0	23.93			
26												
32		219.0	18.50								202.0	16.10
33												
40		203.0							17.55			
45		207.0	17.28	10,040	211.0	17.37	909		16.64	660	163.0	15.29
56	20,400			15,200	151.0	15.88				581	171.0	16.02
57				10,680	159.0	12.92				577	146.0	14.64
58				12,300	194.0	16.11						
59		153.0	13.56					175.5	14.32			
65	8,380	78.0	6.83				1,182	180.0	14.11			
68	10,310	93.0	7.18									
		94.0	7.28									
120										1,065		17.76

### *Postmortem findings*

Gross findings were not important.

#### *Microscopic examination—*

*Heart.* The myocardium of both dogs is normal. In dog 4 there is marked dilatation of the lymphatics of the epicardium. Some of them contain red blood corpuscles. One small artery in the myocardium shows a lesion of the media. Many smooth muscle fibers are densely stained, with pyknotic nuclei. Some of the smooth muscle cells are hyalin and acidophilic. Others stain densely with basic stain and are fragmented. In the adventitia there are many polymorphonuclear leukocytes.

*Lungs.* There are no lesions in the lungs of dogs 2 and 4.

*Spleen.* Dog 2. The follicles are small and inactive. The reticular cords contain very few lymphoid cells. In the sinuses are occasional clumps of densely stained fused red blood corpuscles. There is no erythropoiesis; no megakaryocytes. There are numerous phagocytes containing light yellow globules. The spleen of dog 4 shows some erythropoiesis and numerous megakaryocytes. The sinuses contain clumps of fused red blood corpuscles, as in dog 2. The secondary follicles show moderate activity.

*Liver.* In dog 2 there are many small to very large necroses. The smallest necroses appear in the mid-zonal regions. The larger necroses involve several lobules. These necroses contain very few leukocytes and are therefore regarded as of recent origin. There is fairly marked central fat vacuolization in the lobules throughout the liver. The cells lining the sinusoids are swollen and finely vacuolated. The liver of dog 4 shows no necrosis, no central fat vacuolization. The cells lining the sinusoids are swollen and vacuolated, as in dog 2. The liver cells as a whole contain more basic staining granular material than those of dog 2. In the sinusoids are occasional clumps of fused red blood corpuscles.

*Gall bladder.* The gall bladder from dog 2 is not preserved. That of dog 4 shows lesions in many arteries throughout the thickness of the gall bladder wall of the same nature as the lesions in the artery of the myocardium. These vascular lesions will be described more fully below.

*Pancreas.* The pancreas from each dog is negative. In dog 4 the larger veins contain clumps of fused red blood corpuscles.

*Gastrointestinal tract.* Dog 2. Esophagus, stomach and small intestine, normal. In dog 4 many blood vessels of the stomach

contain clumps of fused red blood corpuscles. In the muscularis there is one medium-sized vein with completely degenerated smooth muscle cells in the media, similar to the lesions of vessels of heart and gall bladder. Otherwise the stomach is normal. Small and large intestine normal.

*Kidneys.* The kidneys from both dogs are essentially normal except for atrophy of the epithelium of many of the distal convoluted tubules, more marked in dog 4. In dog 4 an occasional tubule shows the epithelium to be represented by a very narrow fringe of cytoplasm surrounding nuclei.

*Adrenals.* The adrenals from dog 2 are normal in appearance. Those from dog 4 show slightly less lipid vacuolization of the fascicular cells of the cortex.

*Bladder.* The bladder of dog 2 is normal except for a completely organized thrombosed vein on the external surface. The bladder of dog 4 is normal except for many acute vesicular lesions to be described below.

*Genitalia. Testis*—Dog 2. Immature, normal. *Uterus*—Dog 4. The mucosa is immature but otherwise negative. *Ovary*—Immature but otherwise negative.

*Lymph nodes.* The lymph nodes taken from many sources in both dogs show sinuses containing many red blood corpuscles. All of the lymph sinuses are dilated. There is only a moderate amount of phagocytosis of the red blood corpuscles. The lymphoid follicles are normal. Lymph sinuses and veins show clumps of fused red blood corpuscles. In one lymph node, from dog 4, there is an artery showing the degenerative lesion of the media noted in other arteries.

*Thyroid gland.* From both dogs, normal.

*Skeletal muscle.* That from dog 2 shows no lesion. That from dog 4 shows one vein with degeneration of the smooth muscle cells of the media.

*Aorta.* That from dog 4 is normal.

*Skin.* That from dog 2 is normal.

*Vascular lesions in dog 4.*<sup>2</sup> Degenerative lesions of the media are found in arteries and veins of the myocardium, gall bladder, urinary bladder, and one lymph node. These lesions are characterized by degeneration of smooth muscle cells and various stages in their development are present. The earliest change ap-

<sup>2</sup> The occurrence of clumps of fused red blood corpuscles in the sinusoids of the liver and sinuses of the spleen as an almost constant feature of hypervitaminosis A in rats and guinea pigs should be mentioned here as an early unpublished observation of one of the present authors (S. B. W.).

pears as an homogeneous dense acidophilic staining of the smooth muscle cells. More advanced lesions show infiltration of the media with strands of fibrin, proliferation of the intimal endothelium, and frequently a collection of red blood corpuscles beneath the endothelium. Mitotic figures are present in the adventitia and also in the endothelium. Some small veins show marked thickening as the result of intimal and adventitial proliferation. In such veins, while the lumen remains open, it is narrowed as a result of endothelial proliferation.

Another finding is that of mural thrombi of fused red blood corpuscles. The appearance suggests that the proliferation of the endothelium is the result of these thrombi, inasmuch as many of the thrombi are covered with one or more layers of endothelial cells. Occasionally red blood corpuscles are found intermingled with the proliferated (fibroblastic) cells of the adventitia. In some sections of the wall of the bladder practically every blood vessel shows these lesions. The lesions of the blood vessels of the gall bladder are identical and, also, in the mucosa there are many thrombosed small vessels which have resulted in small infarctions. In addition, many of the involved blood vessels are surrounded by small zones of hemorrhage.

While it seems unwarranted at present to regard these blood vessel lesions as a direct result of excessive vitamin A administration, we are at a loss to account for them, as they are quite different from any infectious lesion of the blood vessels that we know of and also quite different from the blood vessel lesions associated with the allergic reaction.

*Bones.* Based on knowledge of the skeletal changes resulting from vitamin A administration and gained from the study of small animals (Wolbach, '47), we selected for study in longitudinal and cross sections the epiphyseal ends of the tibia, fibula, femur, radius and ulna of dog 2. It should be kept in mind that the results on bone of excessive vitamin A administration are exhibited only in the growing animal and only in regions undergoing growth. A corollary from older studies is that the greater the rate of growth in the region the more marked are the consequences, which, in brief, consist of acceleration of the normal sequences of endochondral bone formation and the remodelling processes.

The effects on the growth sequences, including epiphyseal cartilage sequences and remodelling processes, are of the

same nature as those described for the rat and guinea pig (Wolbach, '47), though of lesser degree, which can be accounted for by the facts that the dogs were two months old when the experiment began and that the dosage of vitamin A was 300 I.U. per gram as compared to the 1,000 to 1,250 I.U. per gram used for rats and guinea pigs. The duration of the experiment on dog 2 was 68 days. With rats, the dosages used resulted, in 6 to 12 days, in the severe lesions including fractures already described (Wolbach, '47).

The epiphyseal cartilages in dog 2 of the regions chosen for study all exhibit complete consumption of fully grown cells, which has resulted in marked narrowing of the cartilage as a whole. Some of the appearances of the cartilage indicate that inanition effects have retarded the growth of the flattened cells in the cartilage columns, but here and there one sees deep penetration of the cartilage and blood vessels (fig. 1). Another indication of rapidity of growth sequences is the persistence of the bony trabeculae of the primary spongiosa.

In the shafts of the bones where remodelling normally takes place there are evidences of great increase in subperiosteal resorption of bone, just as has been described for smaller animals (figs. 2 and 3). There is, on the whole, much less evidence of new bone deposition, although newly deposited bone is present in regions of remodelling in conformity with the normal growth pattern and is much greater than deposition of bone in regions not being remodelled. The lesser amount of new bone formation as compared to rats and guinea pigs is probably the result of two factors—the slower tempo of the experiment and terminal inanition. In all regions of accelerated resorption of bone there is marked proliferation of periosteal cells and considerable hemorrhage, so that the appearances in such locations recall the hemorrhages of scurvy and a deficiency in intracellular matrix formation is to be considered as a possible factor, although it is not reconcilable with the appearances at the epiphyses and the deposit of osteoid wherever required by the normal growth pattern. Accelerated resorption of bone resulting in dimin-

ishing strength of periosteal attachments is a reasonable explanation of hemorrhages, although one we are not yet willing to commit ourselves to as a complete explanation because excessive vitamin A administration results in a depression of prothrombin levels (Light, Alscher and Frey, '44; Walker, Eyllenburg and Moore, '47; Maddock, Wolbach and Jensen, '48).

Cross sections of femur, tibia and fibula reveal accelerated remodelling changes of cortical bone as required by the normal growth pattern. Cross sections of the femur show exaggeration of the flattening of the posterior surface with a great increase in the number of osteoclasts, periosteal proliferation and hemorrhage. The interior of the shaft in this region shows newly deposited osteoid and formation of compact bone in progress. The cortex, anteriorly, of the shaft shows a much less degree of the same process, indicating that at the level chosen the shaft is undergoing diminution in thickness.

The cross section of the tibia likewise shows remodelling sequences greatly in excess of the normal, most marked on the posterior surface and internal and anterior borders.

The most striking evidence of the accelerated remodelling is shown in the cross section of the fibula, of which only a small amount of compact bone remains. On one side, resorption is active. On the opposite side there is a thick layer of newly formed bone and osteoid, features required by the shift in relationships attending growth with increasing separation of tibia and fibula (figs. 4 and 5).

#### DISCUSSION

That characteristic skeletal changes and death as a result of the administration of excessive doses of vitamin A are phenomena present in the dog as well as in other mammals studied was doubted by some of the German workers in this field (Wendt, '35). The present study indicates that with sufficient dosage given over a sufficiently long time, the dog will respond as do the smaller laboratory animals. There is

some evidence to indicate that the dog, because of a possible lessened susceptibility to the effects of high doses of vitamin A, does not possess as delicately balanced a mechanism for the adjustment of vitamin A levels as does the rat or the human (Josephs, '42). The level of 2,420 I.U. per 100 ml of serum encountered during the course of this experiment in an adult dog on a regular diet is in excess of the maximum value of 950 I.U. per 100 ml of serum cited by Josephs in his human case of hypervitaminosis A (Josephs, '44), of the 1,200 reported by Toomey and Morissette ('47) and of the 2,081 reported by Rothman and Leon ('48) in other human cases. The dog is also unusual in his failure to respond to vitamin A administration by heightened cholesterol and phospho-lipid values, a finding noted by Wendt in 1935. Serum lipid rises after vitamin A feeding, either in large or toxic doses, have been noted in the human (Takahashi et al., '25; Harris and Moore, '28; Collazo, Rubino and Varela, '29); the rabbit (Harris and Moore, '28; Bomskov and Seemann, '33); the guinea pig (Collazo and Rodriguez, '33) and the rat (Drigalski, '33). The opposite effect, a fall in cholesterol (Drigalski, '33) and total lipids (Moll et al., '33) in the rat on vitamin A deficient diets, makes the implication that vitamin A is a factor in lipid metabolism a possible hypothesis.

The present study also emphasizes the fact that there are no detectable changes from the standpoint of the blood picture in carbohydrate, nitrogen and mineral metabolism. Except for a slightly heightened calcium level in one dog, the calcium, phosphorus and phosphatase values were essentially normal. Similar results have been noted in the rat (Davies and Moore, '34; Strauss, '34-'35; Cornil, Chevallier and Paillas, '39). Josephs' ('44) case of hypervitaminosis in a child showed normal calcium and phosphorus values but heightened phosphatase values, persisting as long as 6 months after the vitamin had been withheld.

In view of the great skeletal changes resulting from excess vitamin A administration, the absence of changes in calcium, phosphorus and phosphatase values may seem perplexing.

It must be kept in mind that the effect upon the skeleton is one of increased tempo of growth processes in complete conformity with normal growth patterns, in which removal of bone is balanced by new formations of bone and, *a priori*, the rate of metabolic activities resulting therefrom need not be reflected quantitatively in the blood.

It may be suggested that the accelerated rate of consumption of epiphyseal cartilage—essentially an acceleration of skeletal ageing—is accompanied by quantitative changes in the blood of an unknown nature.

#### SUMMARY AND CONCLUSIONS

1. The dog responds, as do other laboratory animals, to excessive doses of vitamin A.

2. This response is marked by loss of appetite, marked loss in weight, characteristic skeletal changes, hyperesthesia and exophthalmos.

3. Laboratory findings, with the exception of marked increases in the vitamin A content of the blood and a late drop in blood lipids (lipid-P and cholesterol), show no significant alterations.

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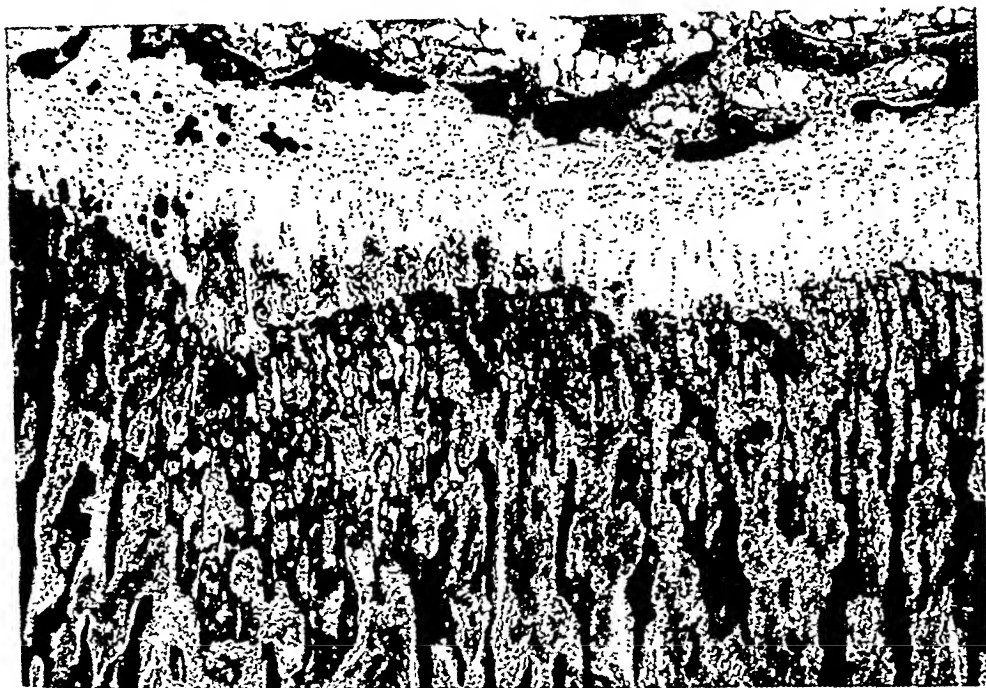
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## PLATE 1

### EXPLANATION OF FIGURES

- 1 Dog 2. Photomicrograph. Epiphyseal cartilage, proximal end of tibia. The evidences of accelerated growth sequences are illustrated by deep penetration of the cartilage by blood vessels, the character of the primary spongiosa, and the penetration of the zone of residual cells by blood vessels from the epiphyseal side.
- 2 Dog 2. Photomicrograph. Proximal ends of the fibula showing complete consumption of active zones of epiphyseal cartilage, resorption of the periosteal bone, and subperiosteal hemorrhage.



1



2

## PLATE 2

### EXPLANATION OF FIGURES

3 Dog 2. Photomicrograph. Distal end of the radius. High power detail of accelerated resorption of bone beneath the periosteum with regions of concurrent bone deposition internally.

4 Dog 2. Photomicrograph. Low power. Cross section of fibula. This shows only a remnant of old bone. The upper right hand border is undergoing resorption and in the lower left there is new bone deposition.



3



4



## EXPERIMENTAL RAT CARIES

### II. LOCATION, SEQUENCE AND EXTENT OF CARIOUS LESIONS PRODUCED IN THE NORWAY RAT WHEN RAISED ON A GENERALLY ADEQUATE, FINELY POWDERED, PURIFIED RATION

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ELEVEN FIGURES

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Experimental caries production in various animal species, which has been more and more successfully accomplished in recent years, will, no doubt, become an increasingly favored *in vivo* research tool in the elucidation of the caries problem.

When faced with the choice of species and dietary regimen, the type and the experimental usefulness of the animal and its carious lesions need careful consideration.

This paper describes a type of caries which, during the past three years, has been produced regularly and at will in common laboratory rats (*Mus norvegicus*) subsisting, from prenatal life to maturity, on a purified, finely powdered ration, adequate in all nutrients currently recognized as essential to growth, reproduction and general health (Sognnaes, '48a, '48b).

#### EXPERIMENTAL

##### *Distribution of the carious lesions*

Under the regimen referred to above, caries has been found to be extremely rare and late in occurrence in the maxillary rat molars, with a ratio of only about one lesion for every 30 found in the mandibular molars of a given litter. The fol-

lowing description will, therefore, be illustrated by the carious lesions occurring in the lower jaw; and, because of the remarkable bilateral symmetry of caries exemplified by figures 1 to 4, it is, for the sake of clarity and brevity, desirable to limit the description to one side of the jaw only; namely, the lower right molars.

The mandibular molars of the rat have, as is indicated by the letters A to F in figures 5 and 6, a total of 6 main fissures: three in the first molar (A, B, C), two in the second (D, E), and one (F) in the third. Out of a total of 15 occlusal cusps, the first molar has 7 (no. 1 to 7, fig. 5), the second molar 5 (no. 8 to 12), and the third molar three (no. 13 to 15). The distribution and size of the fissures and cusps in the three molars seem to justify a ratio of about 5:3:2 between the occlusal area of the first, second and third molars, respectively. This ratio, within a quadrant of the jaw, would be important if one were to desire a finer division of each occlusal surface into the relative number of caries-susceptible squares. A number of fairly complicated scoring methods have been suggested in the past and will be used for refined analysis of experimental data in the future, but an attempt will be made in the present instance to provide a simple grading of 4 well-defined stages of carious destruction, which can be recorded by surface inspection and photography.

In rats subsisting on a purified ration (Sognnaes, '48a), the carious destruction has invariably been found to commence in the occlusal fissures of the mandibular molars. Of the 6 main fissures, the central ones of the first and second molars (B and D in figs. 5 and 6) are, as a rule, the first to show caries, closely followed by the distal fissure of the first molar (C). Thus the pattern of lesions, shown in figures 7 and 8, has quite consistently been the first gross evidence of caries in rats raised on the purified ration. At this step some pigmentation may be seen to extend into the walls of the neighbouring cusps but, where there is no grossly visible loss of surrounding tooth structure (as exemplified by fig. 7), we



may refer to this extent of caries as grade I, which is limited to isolated, non-spreading fissure lesions.

In figure 8 it is obvious that the same fissure lesions have extended to the walls of the surrounding cusps. One of these cusps, no. 7, is completely destroyed; 4 are partly destroyed, two in the first molar, no. 5 and no. 6, and two in the second molar, no. 10 and no. 11. Counting the area of the fissures and cusps involved, we thus arrive at a total of 10 carious areas; that is, about 10% of the total occlusal area of the mandibular right molars. This stage of destruction, which is reached by the beginning breakdown of the tooth substance of the cuspal walls surrounding the fissure lesions, may be classified as grade II.

From this stage the caries may progress to the previously mentioned fissures of the first (A), second (E), and third molars (F), as indicated in figure 9, or this may be preceded by further breakdown of the already existing lesions, as is shown in figure 10. In either case, it is seen that the total extent of caries has increased to a point where about one-third of the total occlusal surface area has been destroyed. Thus in figure 9 all of the 6 fissures are affected, altogether involving about one-third of the total occlusal surface area, and in figure 10 two of the lesions have spread to an extent of about 30% of the total area in view. Such cases, in which from 10 to 30% of the total occlusal area is involved by caries, may be referred to as grade III.

Following this stage, further destruction has been observed to proceed primarily through that part of the cusps which separate the fissures, in which case a lesion beginning in one fissure will tend to fuse with the neighbouring one in the same tooth. Such a course of events has already been noted in its incipient stage in figure 9, where the fissure lesions beginning in fissures B and C meet at the connecting point of cusps no. 5 and no. 6, and in the second molar of figure 10 where cusps no. 10 and no. 11 are broken down to a point where the lesions in fissures D and E are being bridged by caries.

From this stage the second molar is usually the first tooth to be completely destroyed, as is seen in figure 11, followed by the central and distal parts of the first molar. At this stage the third molar as well may exhibit gross destruction, extending to the cusps surrounding the fissure. Hence, in figure 11, more than half of the total area is destroyed by caries, an extent of decay which may be arbitrarily referred to as grade IV. Destruction beyond this stage, or complete breakdown of all the mandibular molars, may be observed after prolonged experimental periods, at which stage the maxillary molars are the only ones left for any further gradation of the caries intensity. The lesions in the mandibular molars are, however, more suitable for the grading of caries in experiments of short duration (two to 5 months). As grade V one may, therefore desire to classify the total destruction of the mandibular molars, which, obviously, would mean the end of masticatory function.

#### COMMENTS

The lesions shown in this series of papers have been produced in rats raised on a finely powdered, purified ration (Sognnaes, '48a) and are not complicated by the mechanical fractures which made differential diagnosis more difficult in the coarse particle type of rat caries introduced by Hoppert, Webber and Canniff in 1931.

All the common lesions have consistently been found to occur in the occlusal surface of the rat molars, in contrast to the varied distribution observed in hamsters (Arnold, '42; Keyes, '46; Sognnaes, '48a). As in hamsters, the rat molars have sufficiently wide and open fissures to permit an early detection of the fissure lesions by careful inspection under a binocular microscope, without destroying the specimen by successive grindings as is necessary in the examination of the deep, narrow fissures of cotton rat molars (Shaw et al., '44). Unaltered by the process of caries examination, the rat jaws may, furthermore, be permanently preserved or im-

mediately prepared for more refined study of their histological, chemical, or physical properties.

It has been determined through histological observations, some of which were presented in the first paper of this series (Sognaes, '48b), that there is a relationship between the extent of the lesions in width and depth. Since significant differences in the extent in width can be judged by a binocular surface inspection, or photography, without destruction of the specimens, it is felt that this means of judging the degree of carious destruction in the Norway rat may be repeated with ease and uniformity by different investigators, whether highly accustomed to dental examinations or not.

The type of caries produced in Norway rats raised on a purified ration has, in addition, proved to be more uniform in character than the lesions produced on other rations and in other rodents. In the first place, the use of a purified ration seems to result in a more even litter-mate distribution of carious lesions than the coarse particle diets used in the past (Sognaes, '41 and others). Secondly, it has been found that male and female rats do not exhibit the sex differences in caries susceptibility which are observed in Syrian hamsters (Keyes, '46; Sognaes, '48a). Finally, and in contrast to both hamsters and cotton rats (Shaw et al., '44), Norway rats reproduce well when subsisting on a purified ration (Sognaes, '48a, '48b), an advantage which is of particular importance for further studies on prenatal nutrition in relation to the caries susceptibility of the offspring.

#### ACKNOWLEDGMENT

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## PLATE 1

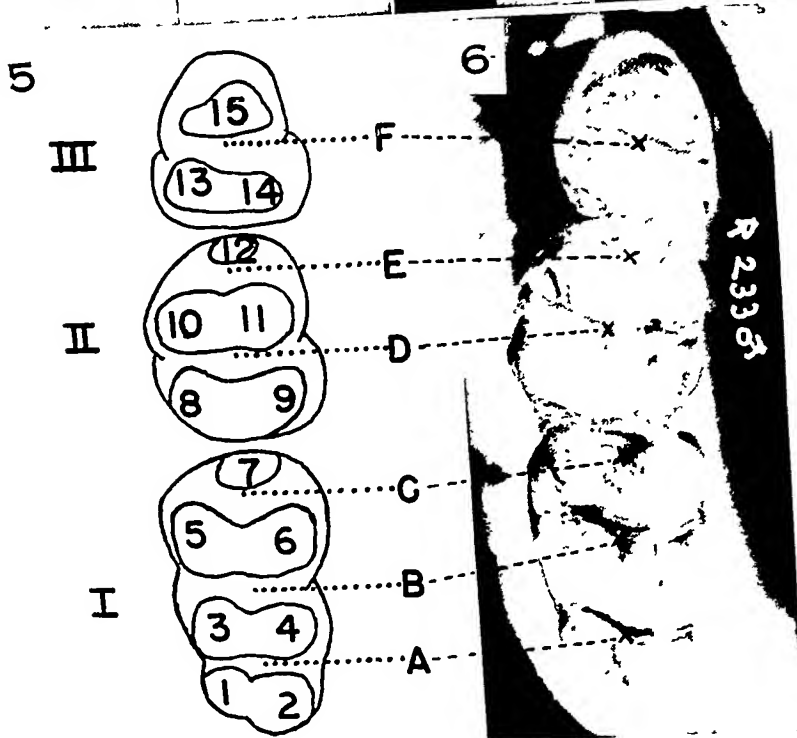
## EXPLANATION OF FIGURES

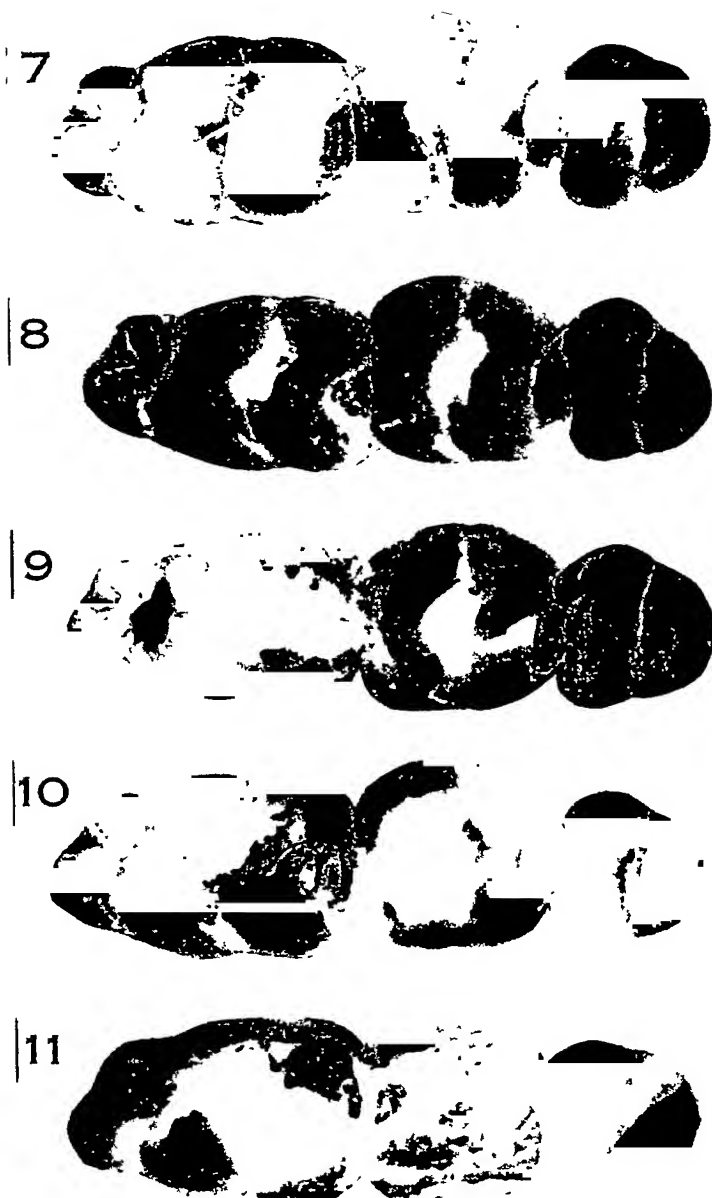
1-11 Mandibular molars of the Norway rat (raised on a finely powdered, purified ration).  $\times 10$ .

1 and 2 Symmetrical bilateral distribution of a moderate degree of carious lesions in the right (1) and left (2) mandibular rat molars.

3 and 4 Bilateral symmetry of extensive carious lesions.

5 and 6 The location of the occlusal fissures and cusps of the three mandibular right rat molars.





7-11 Various degrees of carious destruction. Initial fissure caries, grade I (fig. 7) is followed by involvement of the surrounding cuspal walls, grade II (fig. 8); of the inter-cuspal ridges, grade III (figs. 9 and 10) and, finally, by complete destruction of one or more teeth, grade IV (fig. 11).

# EXPERIMENTAL RAT CARIES

## III. THE EFFECT OF BROMIDE ON EXPERIMENTAL RAT CARIES

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FOUR FIGURES

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Previous investigations (Sognnaes, '48a, '48b, '49) have shown that experimental caries could be produced in the Norway rat when bred by mothers subsisting on a purified ration, while caries did not occur in rats bred by animals fed a stock diet composed of natural food products.

Because the purified ration used in these experiments contained all known nutritional essentials but not all food factors present in natural foods, the question arose of whether some food factor of importance to the teeth might be missing in the caries-producing diet.

When planning further experiments, it first seemed desirable to examine separately some of the volatile elements of the halogen group which could not be recovered by ashing of whole food and whose inhibitory effect upon caries has already been suggested by earlier work reviewed elsewhere (Hodge and Sognnaes, '46).

The present paper deals with the influence on rat caries of one halogen, the caries inhibitory effect of which does not seem to have been tested before; namely, the effect of bromide when added to a purified ration, during tooth development on the one hand and all through the experimental period on the other.

## MATERIALS AND METHODS

The parents of the experimental animals were selected from the same stock of Long Evans strain rats as those used in the first investigations of this series (Soggnæs, '48b, '49). From weaning time to breeding age 5 females and two males, all

TABLE 1  
*Composition of the caries-producing purified ration<sup>1</sup>*

BASAL MIXTURE		SUPPLEMENTS	
	%		4%
Carbohydrate (sucrose, i.e., granulated cane sugar)	67	Liver concentrate	
Protein (casein)	24	<i>Vitamins</i>	
Fat (corn oil)	5	Thiamine	3.5 p.p.m.
Salt mixture	4	Riboflavin	3.5 p.p.m.
<i>Ash analysis of salt mixture</i>		Nicotinic acid	25.0 p.p.m.
Calcium	0.53%	p-Aminobenzoic acid	300.0 p.p.m.
Phosphorus	0.55%	Pantothenic acid	20.0 p.p.m.
Magnesium	0.033%	Pyridoxine	3.5 p.p.m.
Potassium	0.45%	Inositol	1,000 p.p.m.
Sodium	0.44%	Choline	1,000 p.p.m.
Chlorine	0.76%	Vitamin A	30 U.S.P. U/gm
Iron	150 p.p.m.	Vitamin D	1.5 U.S.P. U/gm
Manganese	50 p.p.m.		
Copper	15 p.p.m.		
Cobalt	0.65 p.p.m.		
Iodine	20 p.p.m.		
Zinc	30 p.p.m.		
Sulphur	480 p.p.m.		

<sup>1</sup> The bromine groups of rats were given, in addition to the above ration, 200 µg of bromine as potassium bromide per 10 gm of diet; i.e., 20 p.p.m.

from one litter, were fed the purified ration shown in table 1 with the addition of 200 µg of bromine (as potassium bromide) per 10 gm of the purified diet, i.e., 20 parts per million. This amount, which is the same as that chosen as a high bromine supply in a nutritional study by Winnek and Smith



('37), is nearly the same as that contained in a natural non-caries-producing stock diet.<sup>1</sup>

After three months' subsistence on the bromide supplemented ration, the mating of the above mentioned litter mates resulted in 5 litters totalling 45 animals. These litters were at weaning time divided into two groups. One-third of these rats, i.e., a total of 15 animals, were continued on the bromide supplemented diet (group I). In the other group, an average of two-thirds of each litter, i.e., 30 animals, the addition of bromide was discontinued at that time (group II).

Three months after weaning all animals were sacrificed, except three litter pairs, which were continued on their respective diets for another two months in order to follow further progress of the existing molar lesions and to include some tests of the oral flora. (Data on the latter will be expanded and reported elsewhere.)

After sacrifice of the animals the jaws were fixed in 10% formalin, examined under binocular microscope, upon which examination the caries scoring was based, and finally prepared for histological study.

## RESULTS

The supplement of bromide during tooth development alone did not lessen the high caries susceptibility previously observed in rats bred by mothers subsisting on the purified ration, but continuation of the bromide feeding after tooth eruption did provide some inhibition and delay in the progress of caries.

In table 2 is a comparison of the two groups, one of which was continued (group I) and one discontinued (group II) on the bromide ration after eruption of the molar teeth. After three months, the animals which were continued on the bromide supplemented diet had only half as many carious areas as their litter mates from whose diet the bromide was removed at weaning. Furthermore, three out of the 12 rats in

<sup>1</sup> Purina laboratory chow.

TABLE 2

*The effect on rat caries of bromide added to the purified ration*

GROUP	EXPERIMENTAL CONDITION	LITTER REFERENCE	NO. OF RATS	EXPERIMENTAL RESULTS <sup>1</sup>		
				Molars affected	Carious areas	Caries score
I	Continuous bromide supplement before and after eruption of molars	A	2	1.5	1.5	1.5
		B	3	2.0	2.3	3.0
		C	3	2.6	2.6	4.0
		D	4 <sup>2</sup>	3.2	5.0	7.0
		Average. for all litters		2.5	3.1	4.3
II	Bromide supplement discontinued after eruption of molars	A	4	4.5	6.7	9.5
		B	7	4.0	5.6	7.1
		C	5	4.6	6.6	8.2
		D	6	4.7	7.3	11.0
		E	5	4.8	8.4	14.0
		Average for all litters		4.5	6.8	9.9

<sup>1</sup> Based upon binocular examination ( $\times 30$ ) of molars following sacrifice of the rats three months after weaning.

<sup>2</sup> Photomicrographs in figures 3 and 4 are from the molars of one of these litter mates.

TABLE 3

*Individual litter-mate comparison of caries intensity with and without prolongation of the bromide supplement*

GROUP	EXPERIMENTAL CONDITION	LITTER REFERENCE	EXPERIMENTAL RESULTS <sup>1</sup>		
			Molars affected	Carious areas	Caries score
I	Continuous bro- mide supplement before and after eruption of molars	A	3	3	3
		C	4	5	5 <sup>2</sup>
		E	3	5	7
		Average		4.3	4.6
II	Bromide supple- ment discontin- ued after erup- tion of molars	A	6	8	16
		C	8	9	34 <sup>2</sup>
		E	8	21	46
		Average		16.0	32.0

<sup>1</sup> The rats recorded in this table were sacrificed and examined 5 months after weaning.

<sup>2</sup> The molars (one quadrant of the mouth) of these animals may be compared by means of the gross photographs shown in figures 1 and 2.

group I, one from each of three litters, maintained intact teeth, while all of the 27 rats in group II developed caries. This difference becomes more marked between the litter mates which were sacrificed after 5 months. Thus, table 3 shows that hardly any further progress of the lesions occurred in those rats which were continued on the bromide diet. In the group fed the purified diet without bromide addition, the



Fig. 1 Nonprogressive caries, grade I, in the mandibular left molars of female rat belonging to group I (see table 3) which was fed the purified ration with continuous bromide supplement for 5 months after weaning.

Fig. 2 Progressive caries, grade III, in the mandibular left molars of female litter mate of above, belonging to group II (see table 3), which was fed the purified ration without bromide addition for the same period after weaning.

carious score showed a three-fold increase over the previous level and was 6 times higher than that of group I. It is of interest that the severity of caries was more affected by the bromide than the incidence. The great difference between group I and II is quite consistent in each pair of litter mates, especially after 5 months, as is evident from table 3 as well as from the photographs shown in figures 1 and 2.



Fig. 3 Decalcified paraffin section, Masson stain ( $\times 10$ ), showing the mandibular molars of rat fed the purified ration with the addition of bromide for three months after weaning (see group I, table 2). The carious disintegration is walled off by a considerable layer of secondary dentine, suggesting slow progress of the lesions.

Fig. 4 Serial section from above, Gram stain ( $\times 500$ ), showing undermining caries of distal cusp of first molar with a dense Gram-positive layer of disintegrated dentine lining the cavity and a few areas extending into underlying dentinal tubules.

On the whole, the severity of caries was greater than expected in the animals who received the bromide addition during the period of tooth development only. Indeed, this group developed more, rather than less, caries than rats of the same strain which in the past had been fed the basic purified ration, i.e., without bromide supplement for a comparable period. It may be mentioned that two sisters of the bromide-fed mothers of groups I and II were maintained on the basic purified ration and produced litters (cousins of groups I and II) which, after a comparable experimental period, showed a caries score falling between those of groups I and II. This suggests that bromide is ineffective, if not detrimental, when administered during tooth formation alone, and that the mechanism of action of bromide in reducing caries only appears to operate when the bromide is given after tooth eruption.

#### DISCUSSION

Bromine is not supposed to be a factor nutritionally essential for rats. Winnek and Smith ('37) fed rats a purified, practically bromine-free ration from weaning to 200 days of age. No significant difference in growth or reproduction was found between the bromine-depleted animals and groups fed a supplement of 20 p.p.m. of bromine as potassium bromide. In their experiments they nevertheless noted that the mothers fed the highly purified bromine-free ration did not take sufficient care of their offspring to raise the litters, as a result of which nothing is known about the effect of such a ration upon the future health of the offspring of bromine-deficient rats, i.e., the effect of a deficiency lasting over several generations. Similar criticism may perhaps be made of the negative findings available regarding the nutritional essentiality of other trace elements, such as fluorine.

Although no analysis of the bromine content of the teeth was made, it is interesting that bromine-fed rats had 4 times as high bromine content in their tissues as the animals fed a natural stock diet with similar bromine content (Winnek and Smith, '37). This suggests that bromine in a purified

diet may take the place of some other elements, possibly of other halogens, as is suggested by observations of the chlorine: bromine ratios of blood following bromide injections (Hastings et al., '32). If it is confirmed that animals which receive a bromide supplemented purified ration during tooth formation show an increase rather than a decrease in susceptibility to caries, the possibility may appropriately be considered that bromine can take the place of a factor more essential to the development of caries-resistant tooth structure.

Posteruptively, on the other hand, the presence of bromide did provide some reduction in the extent of caries. If this effect is confirmed, then bromide is the third halogen compound to show a measurable caries-inhibiting effect when added to a caries-producing diet after eruption of the teeth. In searching for a factor which would inhibit oral fermentation, Miller ('38) observed a reduction of the molar lesions in rats resulting from the addition of fluorides and iodoacetate, respectively, to a coarsely ground ration. Because the lesions produced by such a ration are probably mechanical in their initiation, it may, therefore, be assumed that Miller and others using the coarse rations (see review by Hodge and Sognnaes, '46) have observed an inhibition of the progress rather than the initiation of the lesions. Traumatic factors as contributing causes are believed to have been convincingly ruled out in the rat caries reported in this series, as is indicated in part I of this study (Sognnaes, '48b). Even so, the bromide addition to the finely powdered, purified diet also seems primarily to have reduced the progress of the lesions rather than their initiation. After three months both experimental groups were seen to have developed a fair number of lesions, but the marked difference in severity became apparent two months later. While the lesions of the bromide-fed rats were still in an incipient stage their litter mates, after discontinuation of the bromide supplement, developed very severe lesions.

At present it is not known whether the different caries-conducive effects of sucrose, dextrin, starch, and so forth, on the teeth are due to the form of carbohydrates per se or to the presence in higher carbohydrates of non-removable substances. Of such there are many besides bromine, the presence of which does not appear to be a potent enough caries inhibitor to explain the decreasing caries incidence of rats fed sucrose, dextrin, and other higher carbohydrates. It seems certain from the above findings that the presence of bromide does not explain the consistent caries freedom in rats raised on natural stock diets.<sup>2</sup>

In principle it is interesting, however, that a similarity has been found in the caries-inhibiting effects of compounds containing fluorine, iodine and bromine when added to caries-producing diets and fed to rats *after* eruption of the teeth. The effect of bromine appears, on the other hand, to differ from that of fluorine when given before tooth eruption; that is, during tooth formation alone. Fluorine, at least in doses large enough to cause mottling of the enamel, is believed to have some inhibitory effect upon caries even if given during tooth development only. While the mode of action requires more study in the case of fluorine, such a pre-eruptive mechanism does not seem to apply to bromine, is not known to hold for iodine, and is unlikely as regards the more widely distributed chlorine. The ratio of these elements may, however, be important if, as already suggested, the shortage of one may shift the relative retention of another.

Through experimental caries studies using a purified ration there is hope of elucidating these questions. Further work is now in progress to test the period of maximum efficiency and the permanency of the effect of fluorine on experimental rat caries produced on a purified ration. It may well be that the presumably greater caries inhibition of fluorine in contrast to bromine is due to some combination of actions operating both during the period of tooth development as well as post-eruptively in the oral environment.

<sup>2</sup> Such as Purina laboratory chow.

## SUMMARY

1. The absence of caries previously observed in rats bred by mothers subsisting on a natural stock diet, as compared to the extensive caries produced in rats bred on a purified ration, cannot be attributed to the presence of bromine in the stock diet, because the addition of high doses of bromide to the purified ration, during tooth development, failed to provide caries protection.

2. The caries susceptibility observed in rats bred by mothers subsisting on a purified diet is not decreased but appears to be somewhat increased by the addition of bromide to the diet during the period of tooth development alone. The possibility is considered that bromine added to a purified ration may take the place of a factor more essential to the caries resistance of the developing teeth.

3. Caries could be reduced, however, when the bromide supplement was continued after eruption of the teeth. This seems to suggest that one and the same factor (at least when added to a purified diet) may be ineffective or unfavorable to the teeth during their development and still be favorable after tooth eruption.

4. It is suggested that more effective caries inhibitors may be found in food factors which exert a favorable action during tooth development as well as posteruptively.

## ACKNOWLEDGMENTS

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# THE URINARY EXCRETION OF PENTOSE- AND PHOSPHORUS-CONTAINING COMPLEXES IN NUTRITIONAL MUSCULAR DYSTROPHY

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The constant urinary excretion of ribose-phosphorus-containing complexes by patients with pseudohypertrophic muscular dystrophy has recently been reported from this laboratory (Minot et al., '49). Since the report by Goettsch and Pappenheimer ('31), extensive work has repeatedly confirmed the observation that muscular weakness and deterioration histopathologically similar to progressive muscular dystrophy in man is produced when young animals are maintained on a diet deficient in vitamin E. Both conditions are characterized by excessive creatinuria, and it seemed of interest to determine whether animals with nutritional dystrophy also excrete pentose-phosphate complexes. The present paper is a report of our investigations in regard to these constituents.

## EXPERIMENTAL

Rabbits varying from 6 to 10 weeks of age at the start of the experiment were used as subjects. They were fed Goettsch and Pappenheimer's E deficient dystrophy-producing diet

<sup>1</sup> Some of the data contributed to this paper by this author was also incorporated in a thesis submitted by him for partial fulfillment of the requirements for a degree of Master of Science from Vanderbilt University.

number 13. To eliminate dietary changes other than the presence or absence of vitamin E, the same food mixture was fed throughout the control and experimental periods. For the control period the treatment of the diet with ether and ferric chloride to destroy vitamin E was omitted, but it was included in the preparation of all food for the experimental period.

In order to avoid the danger of obtaining urine contaminated with particles of food, no attempt was made to collect complete 24-hour specimens of urine. Single samples of urine for chemical studies were obtained by gentle pressure over the bladder or more often by catheterization. Since previous experience had demonstrated the rapid spontaneous disintegration of the complexes in which we were interested, it seemed unwise to carry out our studies of the partition of urinary phosphates on specimens of urine which had been retained in the urinary bladder for variable and unknown periods of time. Hence the plan adopted was to empty the bladder by catheterization, using the urine thus obtained for creatine and pentose studies, while phosphate partition studies were carried out immediately after a sample was obtained by a second catheterization two hours after the first.

The chemical methods employed were those which have been described in greater detail in our earlier paper (Minot et al., '49). Following preliminary treatment with yeast and Lloyd's reagent to remove glucose and other interfering substances (Van Slyke and Hawkins, '29), 10 drops of urine were tested with Benedict's qualitative sugar reagent. At first the osazone test was carried out on many samples of treated urines even though they failed to reduce Benedict's solution. Later, however, since these attempts always gave negative results, such samples as failed to reduce copper after heating for 45 min. in a boiling water bath were considered to be free of detectable amounts of pentose. Samples giving positive reduction tests were treated with phenylhydrazine for the formation of osazones. Phosphorus was determined on fresh two-hour samples of urine by the method of Lowry and Lopez ('46). Three types of determinations were run: (a)

preexisting inorganic phosphorus; (b) phosphorus released by 7-minute hydrolysis with 1 N hydrochloric acid; and (c) total urinary phosphorus following wet ashing with sulphuric and nitric acids. Creatine and creatinine determinations by the method of Folin ('14) were also carried out because creatinuria has become so well established as a criterion of the onset and progression of nutritional muscular dystrophy (Verzar, '39; Mackenzie and McCollum, '40).

Objectively the young rabbits were lively, ate well and gained weight during the first three or 4 weeks on the E-deficient diet. The first indications of deficiency were a cessation in weight gain, some loss of appetite, and a tendency to less activity. From this point on deterioration was progressive and fairly rapid. Increasing muscular weakness and wasting developed and there was a considerable loss in weight. One rabbit died prematurely at the end of 4 weeks when only early signs of muscular deterioration were apparent. At autopsy this animal was found to have an intestinal obstruction. Four others, after eating the deficient diet for periods varying from 6 to 9 weeks, became helpless and either died or were sacrificed when in a moribund condition. At autopsy, in addition to the extensive typical changes in skeletal muscles, two rabbits that died spontaneously showed acutely dilated hearts, a finding we have seen frequently in E-deficient animals and one which has been described by Houchin and Smith ('44). In one instance (rabbit 2), when signs of muscular weakness had first become unmistakable, vitamin E was restored to the diet and supplemented with parenteral injections of  $\alpha$ -tocopherol phosphate. All signs of muscular weakness soon disappeared and the rabbit is still living and well several months after the experiment.

Typical chemical data obtained during the control and experimental periods are presented in table 1. Many more confirmatory repetitions of the same studies were, however, carried out at approximately three-day intervals throughout the experiments. In no instance was any pentose detected either by reduction test or by the formation of osazone dur-

TABLE 1

RABBIT NO.	TOTAL CREA- TININE AS CREATINE	PENTOSE		TOTAL PHOS- PHORUS IN 2-HR. SAMPLE	DISTRIBUTION OF URINARY PHOSPHORUS			CONDITION OF EXPERIMENTAL SUBJECT	
		Reduc- tion test	Oxazone		Pre-existing inorganic P	"7 minute" P			
			%			%	%		%
1.	6.2 18.6	- +	- +	7.0 5.3	96.3 84.1	3.7 15.9	0 11.5	Control 4 weeks on deficient diet, losing weight, died pre- maturely of intestinal obstruction	
2.	8.2 12.5 33.0 14.9	- - + -	- - + -	5.8 15.5 5.4 3.3	95.7 89.7 88.1 92.7	4.3 10.3 11.9 7.2	0 0 8.1 0.1	Control 3 weeks on deficient diet, gaining weight 9 weeks on deficient diet, definite weakness 1 month after restoration of vitamin E	
3.	5.5 21.8 56.5	- - +	- - +	7.3 2.3 0.6	97.2 92.9 67.2	2.8 7.1 32.8	0 0.4 26.9	Control 3 weeks on deficient diet, no symptoms 5 weeks on deficient diet, extreme weakness, eating nothing, died 2 days later	
4.	10.0 7.7 39.6 45.4	- - + +	- - + +	2.6 13.9 11.1 3.2	95.9 94.2 93.7 91.6	4.1 5.8 6.3 8.4	0 0 5.4 3.4	Control 3 weeks on deficient diet, gaining weight 5 weeks on deficient diet, has ceased to gain 7 weeks on deficient diet, marked weakness, not eating	
5.	10.3 20.3 .. 58.5	0 + + +	0 + + +	11.9 5.2 7.5 2.4	95.8 92.7 93.0 91.2	4.2 7.3 7.0 8.8	0 1.0 4.8 5.7	Control 4 weeks on deficient diet, appears well 6 weeks on deficient diet, slight weakness, loss of ap- petite 9 weeks on deficient diet, extreme weakness and emaci- ation, died 4 days later	
6.	2.1 7.8 10.2 34.5 44.0	0 0 0 + +	0 0 0 + +	6.6 12.4 8.4 11.0 6.5	94.7 90.3 93.6 95.5 91.3	5.3 9.7 6.4 4.5 8.7	0 0 0 3.6 3.1	Control 3 weeks on deficient diet, gaining weight 5 weeks on deficient diet, appears normal 8 weeks on deficient diet, some weakness, losing weight 9 weeks on deficient diet, very weak, eats nothing	

ing the control periods or the first week or two that the rabbits ate the E-deficient diet. Studies of the partition of urinary phosphate during this same interval served to detect no readily hydrolyzable (so-called 7-minute) phosphorus, although three to 5% of the total phosphorus was present in organic combination. Approximately coincident with the first objective signs of deficiency both pentose and small amounts of readily hydrolyzable phosphate appeared in the urine, and these positive findings persisted until the end of the experiment in the 5 untreated rabbits. A considerable increase in creatinuria usually preceded by several days the presence of detectable amounts of pentose-phosphorus-complexes in the urine. In the later stages of deficiency the excessive creatinuria so characteristic of muscular dystrophy was apparent in every instance. The typical changes in creatine, pentose, and phosphorus excretion which were observed with the onset of symptoms in rabbit 2 returned to normal after the restoration of vitamin E.

For purposes of further identification, several samples of crude osazones prepared from the urine of these rabbits were pooled and repeatedly recrystallized. The purified osazone melted at 156.0°C. and no reduction of melting point was noted when the osazone from rabbit urine was mixed with pure ribosazone or with that obtained from the urine of patients with pseudohypertrophic muscular dystrophy. This and other incidental evidence in regard to the precipitability of pentose complexes with mercury in freshly obtained urine, as described in our earlier paper, all indicated that the pentose-phosphorus-complexes excreted in the urine in both nutritional and clinical muscular dystrophy are probably the same complex.

#### COMMENT

At the present time it is equally impossible to evaluate in nutritional and in clinical muscular dystrophy the significance of the urinary excretion of what appear to be pentnucleotides. One possibility is that the positive urinary findings are merely

evidence of a release of cellular constituents from injured cells into the blood stream and thence into the urine. If so, it must probably be granted that the injury need not be of a specific type, for the deterioration in experimental dystrophy apparently results from a lack of vitamin E, whereas no such deficiency is demonstrable in clinical muscular dystrophy (Minot and Frank, '44). On the other hand, if one indulges in the speculation that some inborn or acquired error in metabolism may be responsible for both the muscular deterioration and the abnormal urinary constituents in clinical dystrophy, the appearance of the same abnormalities in experimental dystrophy might be taken to indicate a failure in the same metabolic step in the two conditions. On this basis, the hypothetical step involved would be one for which vitamin E is essential and which fails when the vitamin is withheld, but which may also fail in the presence of adequate amounts of vitamin E from other, as yet unrecognized, causes.

#### SUMMARY AND CONCLUSIONS

Studies have been carried out on urines obtained from 6 young rabbits with muscular dystrophy produced by a dietary lack of vitamin E.

Control studies were negative. With the onset of signs of muscular dysfunction, pentose and readily hydrolyzable organically bound phosphate appeared simultaneously in the urine. The isolation and identification of pentose as an osazone and the liberation of bound phosphate by 7-minute hydrolysis with 1 N HCl was considered chemical evidence of the presence of pentose-phosphorus-containing complexes apparently identical with those reported in clinical cases of progressive muscular dystrophy.

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# THE ESSENTIAL AMINO ACID (EXCEPT TRYPTOPHAN) CONTENT OF COLOSTRUM AND MILK OF THE COW AND EWE<sup>1</sup>

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Although there has been considerable work done on milk proteins, a review of the literature revealed only a few reports on the essential amino acid content of whole milk protein, and limited comparative data on the amino acid composition of the milk and colostrum of different species.

The amino acid content of human and cow's milk has been compared by Plimmer and Lowndes ('37), Beach et al. ('41), Williamson ('44) and Block and Bolling ('46). The whole milk data of Plimmer and Lowndes, Beach et al., and Williamson were derived from analysis of casein and other protein fractions. Block and Bolling's work included an analysis of human colostrum. Microbiological data on the amino acid content of fresh and processed whole cow's milk have recently been reported by Hodson and Krueger ('46, '47). Cow's milk and colostrum have been analyzed for tryptophan (Sutton and Esh, '48; Esh and Sutton, '48).

Colostrum appears to provide special nutrients which enhance the chance of survival of the newborn animal. It is richer in certain vitamins than milk (Dann, '33; Semb et al., '34; Pearson and Darnell, '46; Parrish et al., '49). Not only is the protein content greater, but the protein distribution

<sup>1</sup>Acknowledgments are made to Mary Trant, Shirley Dieterich, Lila Corley and Patricia Sparks for technical assistance with the work.

is entirely different from that of milk (Crowther and Rastriek, '16; Parrish et al. '48). Immune proteins are known to be transferred from the mother to her offspring through colostrum. The amino acid composition of these immune proteins has been determined by Smith et al. ('46, '47). In view of these considerations, and the fact that the biological value of a protein is largely determined by its essential amino acid content, it appeared desirable to carry out a comparative study of the essential amino composition of the total protein of the colostrum and milk of the cow and sheep. Information on the amino acid composition of colostrum may find application in the nutrition of calves and lambs, especially where substitutes for milk are used.

## EXPERIMENTAL

### *Methods*

Samples of colostrum were obtained from the cows within 12 hours after parturition and from the ewes within three hours after lambing. Milk was collected after the animals had been lactating for 30 days or longer. In the case of the cows, colostrum and milk samples were obtained from the same animal. The death of a few of the lambs made it impossible to obtain paired colostrum and milk samples in all cases. When this happened, milk was obtained from another ewe of the same breed which had been nursing a lamb for 30 days. The samples were immediately analyzed for total nitrogen, and acid hydrolysates were prepared within 24 hours.

Total nitrogen was determined by semi-micro Kjeldahl procedure. Crude protein was calculated by multiplying the nitrogen value by the factor 6.38.

Twenty milliliters of colostrum or 50 ml of milk were diluted with an equal volume of 12 N HCl, and then 6 N HCl was added until a total volume of 100 ml per gram of crude protein was obtained. This mixture was refluxed for 24 hours. After the removal of the excess acid by vacuum distillation,

the hydrolysates were diluted to 100 ml, filtered and stored in a refrigerator for amino acid analysis.

Microbiological methods of amino acid assay were employed. Valine, leucine, and isoleucine were determined with *Lactobacillus arabinosus* 17-5 (Kuiken et al., '43). *Leuconostoc mesenteroides* P-60 was used to determine methionine (Lyman et al., '46), arginine, lysine and phenylalanine (Kuiken and Lyman, unpublished methods). *Streptococcus faecalis* R was used to determine threonine and histidine (Lyman et al., '47). L amino acid standards were used in the assays for leucine, isoleucine, arginine, lysine and histidine; DL standards were used in the other assays.

#### RESULTS AND DISCUSSION

Data for essential amino acids (except tryptophan) found in cow's colostrum and milk are given in tables 1 and 2, respectively. The data are expressed in terms of the amino acid content of the crude protein, which provides the nutritionist with the most useful description of the relative distribution of amino acids in food products. The same data for ewe's milk are given in tables 3 and 4. Table 5, which gives the ratio of the amino acid content of milk protein to that of the protein of colostrum, was prepared to facilitate comparison of these data.

Two major differences in the relative distribution of amino acids are to be noted. Milk protein from each of these animals contains about 1.4 times the amount of methionine found in the protein of colostrum but only about 0.6 as much threonine. In the case of the other amino acids the relative proportions are more nearly equal. It will be noted that an essentially identical relationship exists between the relative proportions of these amino acids in milk and colostrum for both animals. The work of Block and Bolling ('46) suggests a similar relationship between colostrum and milk in humans. According to their data, a typical mother's milk protein contained 1.3 times as much methionine as a colostrum protein sample, and 0.9 times as much threonine. These differences

TABLE 1  
*Essential amino acid (except tryptophan) content of colostrum of the cow*  
 (Amino acid content expressed as per cent of crude protein)

ANIMAL NUMBER	BREED	CRUDE PROTEIN (N x 6.38)	AMINO ACIDS								
			Arginine	Histidine	Isoleucine	Leucine	Lysine	Methionine	Phenyl- alanine	Threonine	Valine
1	Holstein	13.33	% 4.16	% 2.35	% 4.87	% 9.44	% 8.06	% 1.76	% 4.60	% 7.01	% 8.29
2		14.16	4.30	2.22	4.81	9.49	8.19	1.76	4.51	7.36	8.14
3		18.37	4.68	2.50	5.14	9.74	8.29	1.97	4.93	7.41	8.24
4		15.89	4.34	2.23	4.62	8.97	8.52	1.74	4.60	6.87	7.26
5		15.90	4.15	2.59	4.85	9.22	7.98	2.08	4.58	6.58	7.68
6		16.69	4.41	2.49	4.44	8.67	7.79	1.80	4.40	7.14	7.64
7		10.62	3.79	2.50	4.73	8.77	7.80	1.93	4.31	6.22	7.51
8	Jersey	11.42	4.43	2.31	5.03	9.51	8.42	1.78	4.64	7.67	8.01
9		12.95	4.02	2.43	4.68	8.49	7.56	1.86	4.22	6.28	7.30
10		25.23	4.02	2.36	4.45	8.72	8.05	1.83	4.27	7.25	7.75
11		11.37	4.03	2.33	4.49	8.56	7.63	2.16	4.38	6.84	7.34
12		14.07	4.51	2.64	4.41	9.12	7.88	2.03	4.50	6.95	7.66
Average $\pm$			4.24	2.41	4.71	9.06	8.01	1.89	4.49	6.96	7.73
Standard error			$\pm 0.073$	$\pm 0.038$	$\pm 0.069$	$\pm 0.122$	$\pm 0.087$	$\pm 0.052$	$\pm 0.057$	$\pm 0.128$	$\pm 0.104$

TABLE 2  
Essential amino acid (except tryptophan) content of milk of the cow  
(Amino acid content expressed as per cent of crude protein)

ANIMAL NUMBER	BREED	CRUDE PROTEIN (N x 6.36)	AMINO ACIDS									
			Arginine	Histidine	Isoleucine	Leucine	Lysine	Methionine	Phenyl- alanine	Threonine	Valine	
		%	%	%	%	%	%	%	%	%	%	
1	Holstein	2.85	3.34	2.53	5.59	9.16	8.38	3.01	4.70	4.01	7.03	
2		3.16	3.20	2.56	5.36	8.89	8.11	2.75	4.57	4.28	7.27	
3		3.12	3.36	2.52	5.67	8.98	8.43	2.78	4.54	4.40	6.90	
4		2.60	3.66	2.86	6.11	9.98	9.55	2.75	5.01	4.55	7.23	
5		3.05	3.42	2.69	5.98	9.34	8.54	2.42	4.83	4.44	6.72	
6		3.28	3.54	2.63	5.59	8.89	8.25	2.57	4.88	4.02	7.01	
7		3.08	3.44	2.74	6.04	8.94	8.62	2.37	4.76	4.23	7.04	
8	Jersey	4.29	3.58	2.81	6.29	9.32	8.90	3.06	4.73	4.53	7.49	
9		3.53	3.63	2.78	5.93	9.19	7.87	2.41	4.69	4.41	6.87	
10		3.84	3.64	2.80	6.18	9.39	9.19	2.66	4.86	4.48	7.41	
11		4.09	3.56	2.72	5.76	9.31	8.62	2.58	4.61	4.39	7.29	
12		3.86	3.81	2.78	5.78	8.96	7.80	2.42	4.81	4.35	7.20	
Average $\pm$			3.52	2.70	5.86	9.20	8.52	2.65	4.75	4.34	7.12	
Standard error			$\pm 0.048$	$\pm 0.035$	$\pm 0.080$	$\pm 0.090$	$\pm 0.148$	$\pm 0.066$	$\pm 0.040$	$\pm 0.051$	$\pm 0.067$	

TABLE 3  
Essential amino acid (except tryptophan) content of colostrum of the ewe  
(Amino acid content expressed as per cent of crude protein)

ANIMAL NUMBER	BREED	CRUDE PROTEIN (N x 6.38)	AMINO ACIDS									
			Arginine	Histidine	Isoleucine	Leucine	Lysine	Methionine	Phenyl- alanine	Threonine	Valine	
		%	%	%	%	%	%	%	%	%	%	
1	Delaine Merino	15.82 <sup>1</sup>	4.25	2.31	4.44	9.24	7.48	1.58	4.36	7.44	8.19	
2		20.38	4.06	2.25	4.56	9.35	7.85	1.74	4.30	7.04	8.12	
3		19.87	3.93	2.38	4.53	9.00	7.63	1.48	4.11	7.56	7.96	
4		24.93	4.57	2.51	4.87	9.74	8.46	1.62	4.56	8.14	9.61	
5	Dorset	17.63 <sup>1</sup>	3.80	2.34	4.38	9.29	7.71	1.70	4.21	6.89	7.85	
6		14.51 <sup>1</sup>	3.94	2.59	5.25	9.82	8.15	2.26	4.68	6.09	7.67	
7		22.68 <sup>1</sup>	3.78	2.46	4.90	10.03	8.16	1.87	4.64	6.44	8.14	
8	Ram- bouillet	11.31 <sup>1</sup>	3.84	2.68	4.85	9.56	7.70	2.05	4.48	6.86	7.95	
9		18.96 <sup>1</sup>	3.62	2.77	5.28	10.09	8.20	2.28	4.72	5.98	7.89	
10		20.28	3.84	2.59	4.65	10.01	7.81	2.14	4.78	6.43	7.93	
11	Suffolk	14.44	4.00	2.63	5.19	10.38	8.48	2.15	4.49	6.97	7.87	
Average $\pm$			3.97	2.50	4.81	9.66	7.97	1.90	4.48	6.89	8.11	
Standard error			$\pm 0.085$	$\pm 0.050$	$\pm 0.098$	$\pm 0.130$	$\pm 0.123$	$\pm 0.087$	$\pm 0.066$	$\pm 0.196$	$\pm 0.157$	

<sup>1</sup> Colostrum and milk samples were not obtained from the same animal.



TABLE 4  
Essential amino acid (except tryptophan) content of milk of the ewe  
(Amino acid content expressed as per cent of crude protein)

ANIMAL NUMBER	BREED	CRUDE PROTEIN (N x 6.38)	AMINO ACIDS									
			Arginine	Histidine	Isoleucine	Leucine	Lysine	Methionine	Phenyl- alanine	Threonine	Valine	
		%	%	%	%	%	%	%	%	%	%	
1	Delaine Merino	4.71 <sup>1</sup>	3.05	2.73	5.50	9.80	9.11	2.48	4.64	4.34	7.80	
2		4.58	3.28	2.70	5.64	9.71	9.09	2.50	4.75	4.44	7.33	
3		5.21	3.15	2.82	5.70	9.82	8.52	2.67	4.67	4.63	7.33	
4		4.58	3.11	2.90	5.46	9.79	8.52	2.37	4.75	4.29	7.58	
5	Dorset	4.98 <sup>1</sup>	3.13	2.80	5.54	9.88	9.01	2.48	4.63	4.39	7.88	
6		4.35 <sup>1</sup>	3.44	2.72	5.55	9.69	8.11	2.69	4.78	4.47	6.93	
7		4.98 <sup>1</sup>	3.26	2.75	5.51	10.11	8.50	2.64	4.61	4.41	7.44	
8	Ram- bouillet	4.90 <sup>1</sup>	3.08	2.85	5.61	9.71	8.24	2.69	4.92	4.63	8.16	
9		4.36 <sup>1</sup>	3.48	3.03	6.02	10.41	8.33	2.76	5.12	4.43	7.44	
10		4.58	3.42	2.68	5.39	9.48	8.50	2.51	4.62	4.32	7.43	
11	Suffolk	4.63	3.48	2.81	5.68	9.46	8.74	2.59	4.79	4.25	7.67	
Average ±			3.26	2.80	5.60	9.80	8.61	2.58	4.75	4.42	7.54	
Standard error			± 0.051	± 0.031	± 0.051	± 0.081	± 0.103	± 0.038	± 0.046	± 0.037	± 0.099	

<sup>1</sup> Colostrum and milk samples were not obtained from the same animal.

reflect the change in protein distribution which occurs during the transition from colostrum to milk production. The immune proteins characteristic of bovine colostrum may represent as much as 50 to 60% of the total protein in colostrum (Smith, '48). Such proteins as a class representing a number of species have a very high threonine content, ranging from 7.4 to 11.1% (Smith and Greene, '47). On the other hand, they contain from 0.7 to 1.2% methionine as compared with about 2.5% in casein (Lyman et al., '46; Hodson and Krueger, '46).

TABLE 5

*Ratios which characterize the relative amino acid composition of milk and colostrum proteins*

AMINO ACID	RATIO OF AMINO ACID CONTENT OF MILK PROTEIN TO THAT OF COLOSTRUM PROTEIN		RATIO OF AMINO ACID CONTENT OF COW'S MILK PROTEIN TO THAT OF EWE'S MILK PROTEIN
	Cow	Ewe	
Arginine	0.83	0.82	1.08
Histidine	1.12	1.12	0.96
Isoleucine	1.24	1.16	1.05
Leucine	1.02	1.02	0.94
Lysine	1.06	1.08	0.99
Methionine	1.40	1.36	1.03
Phenylalanine	1.06	1.08	1.00
Threonine	0.62	0.64	0.98
Valine	0.92	0.93	0.94

The ratios of the amino acid content of cow's milk protein to ewe's milk protein are also presented in table 5. It is evident from a nutritional point of view that the amino acid patterns of these two groups of mixed proteins are essentially identical. From a consideration of amino acid data alone, it would appear that these proteins are of equal nutritional value.

Relatively minor individual variations were observed in the amino acid patterns of milk and colostrum from different animals. The extreme values in almost every case fell well within 10% of the mean. Although the data are grouped according to breed, the authors do not interpret these individual

variations in terms of breed. They probably reflect minor variations among individual animals in the relative proportions of particular proteins.

The authors' data relating to the essential amino acids in cow's milk are in general agreement with values previously reported.

#### SUMMARY

Colostrum and milk of the cow and ewe were assayed for arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine and valine by microbiological methods. The milk protein from both species contained about 1.4 times as much methionine as the protein of colostrum, but only 0.6 times as much threonine. Much smaller differences were noted in the relative proportions of the other amino acids. The amino acid patterns of the milk and colostrum of the two species are very similar.

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# THE NUTRITIVE VALUE OF CANNED FOODS<sup>1</sup>

## I. AMINO ACID CONTENT OF FISH AND MEAT PRODUCTS

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This is the first portion of the report of an inter-laboratory amino acid assay project undertaken at the University of California at Los Angeles and the University of Wisconsin. The objects of this research were: (a) to carry out a rather extensive survey of the amino acid content of some common protein foodstuffs; (b) to establish the reliability of microbiological assay techniques; and (c) to obtain information concerning the extent to which canning may affect the amino acid content of the proteins in canned foods. The results of preliminary experiments and data on those amino acids and samples not investigated in the Wisconsin laboratory are given in this part of the report. Experimental studies duplicated in the two laboratories and those carried out only at the University of Wisconsin are presented in the second part (Neilands et al., '49).

A total of 74 individual samples<sup>2</sup> were assayed for the 10 essential amino acids in the two laboratories. Of this total,

<sup>1</sup> Paper 59. This work was supported in part by funds provided by the National Canners' Association and the Can Manufacturers' Institute, and is No. XXXIII in the series of the nutritive value of canned foods. The authors are indebted to L. E. McClure and Dolores Fickes, who determined moisture, ash and nitrogen. For paper 58, see Dunn et al. ('49b).

<sup>2</sup> All samples were obtained through the courtesy of the National Canners' Association, the American Can Company and the Continental Can Company. Complete details on the sources of the fish and meat products, the methods of collection and preservation, and other matters are available in the files of the National Canners' Association. Many of these data have been omitted from the present paper in order to conserve space.

41 samples were assayed in both laboratories, 20 samples were assayed only at the University of Wisconsin and 16 samples were assayed only at U.C.L.A.<sup>3</sup> The additional amino acids aspartic acid, glutamic acid and glycine were determined in all of the samples assayed by the present investigators. Proximate analyses were made in both laboratories.

#### EXPERIMENTAL

Preliminary experiments were run on fresh sardines to determine the adequacy of preservation processes and microbiological assay methods. The heads, tails and viscera were removed within a few hours after the fish were caught. The edible portions were homogenized in a Waring Blendor and frozen in dry ice. This blended material was brought to the U.C.L.A. laboratory the same day. Aliquots of the mixture were: (a) hydrolyzed with acid or base; (b) lyophilized and stored at room temperature; and (c) stored frozen at dry ice temperature.

Samples of the hydrolyzed, lyophilized and frozen (packed in dry ice) materials were forwarded to the Wisconsin laboratory where the 10 essential amino acids were determined by the methods described in part II of this report. After 44 days of storage all of these materials were assayed for 13 amino acids at U.C.L.A. No significant differences (except for methionine) in the percentages of amino acids were observed between the hydrolyzed fresh material and that stored in the lyophilized or frozen state. Therefore, freezing, shipping and storing at dry ice temperature were adopted as a general procedure in handling samples of fresh, raw materials.<sup>4</sup>

<sup>3</sup> The apparent discrepancy in the total number of samples is due to the fact that samples SP1P, SP2P and SP3P (shrimp) are included in both of the groups analyzed separately at each laboratory rather than in the common group. The reason for this is that these particular samples were accidentally drained in the Wisconsin laboratory and only the solid portions assayed.

<sup>4</sup> All of the fish and meat products assayed for amino acids were furnished by the National Canners' Association from raw materials collected for this purpose.

The fresh materials were cleaned and trimmed and the edible portions were put into cans and processed according to the usual commercial cannery procedures. Twenty-four to 36 cans, numbered consecutively for later identification, were collected for each type of material (only 4 cans of whole ham were provided). When raw as well as heat processed samples were to be studied, the even numbered cans were frozen immediately after sealing by packing them in dry ice and the odd numbered cans were sent to the retorts for heat processing. A few of the samples of tuna were precooked for three to 4 hours at 100 to 103°C. in accordance with commercial preparatory methods, but not heat processed in the cans. These samples were preserved at dry ice temperature and subjected to the same treatment as the raw samples. Representative cans from more than half of the samples were made available to both laboratories and the remaining samples were supplied to one or the other of the two laboratories. All raw and precooked samples were shipped in insulated containers packed with dry ice. At U.C.L.A. these samples were stored at dry ice temperature until assayed. The heat processed samples were shipped without refrigeration and those received at U.C.L.A. were stored at room temperature in the original sealed containers until ready for use. The samples analyzed are designated throughout this and the following paper by a uniform code system. The code designation consists of: (a) two capital letters indicating the type of product; (b) a number to distinguish between different samples of the same product packed at different canneries; and (c) a final "R," "RC," or "P" indicating that the material was raw, precooked, or heat processed, respectively.

Code letters for the various products and scientific names of the fish studied are as follows: MA, Atlantic mackerel, *Scomber scombris*; MP, Pacific mackerel, *Pneumatophorus diego*; SO, Atlantic sardines in oil, *Clupea harengus*; ST, Pacific sardines in tomato sauce, *Sardinops caerulea*; FA, Atlantic fish flakes, *Gadus callarius* (cod) and *Melanogrammus aeglefinus* (haddock); SC, salmon, *Oncorhynchus tsha-*

*wytscha*; SH, spiced ham; WH, whole ham; TO, tuna in oil, *Germo*, *Thunnus*, and *Neothunnus* species; RB, beef; and SP, shrimp. Thus, MA1R, for example, designates a sample of raw Atlantic mackerel, and SC2P a sample of heat processed salmon.

With the single exception of the shrimp samples, all analyses were carried out on the entire can contents, which included not only the actual fish or meat but also, in the case of most of the products, various additives such as water, oil, tomato sauce, salt, spices, and sugar. For this reason the proximate composition of each sample was determined, and the amino acid values found were expressed as a percentage of the crude protein present.

The sampling and assay procedures employed in the U.C.L.A. laboratory are described in the following paragraphs.

The contents of 6 cans of material (one can of whole ham) were weighed, ground in a meat grinder and mixed well to insure representative sampling. A 300-gm aliquot of the mixture was homogenized in a Waring Blendor, using measured amounts of water when necessary to facilitate blending. Aliquots were weighed immediately for acid and alkaline hydrolysis and the remaining homogenate was packed in dry ice for storage until moisture, ash and nitrogen could be determined. Preliminary to the acid hydrolysis, 50 gm of the homogenate were allowed to digest for several days with 100 ml of 12 N HCl at room temperature. Fifty milliliters of water were added and the mixture was refluxed for 20 hours on an oil bath, cooled and filtered. The relatively small amount of residue on the filter paper was washed well with distilled water. The filtrate and washings were combined, made up to volume (500 ml) and stored in the refrigerator (at 4°C.). An appropriate aliquot was neutralized and diluted to the volume required for the assay. The assay procedures were those described previously (Dunn et al., '49a). Aspartic acid, glycine, lysine and phenylalanine were determined with *Leuconostoc mesenteroides* P-60; arginine with *Lactobacillus*



casei; glutamic acid, isoleucine, leucine and valine with *Lactobacillus arabinosus* 17-5; and histidine, methionine and threonine with *Lactobacillus fermenti* 36.

For alkaline hydrolysis a 10-gm aliquot was mixed with a solution of 10 gm of NaOH in sufficient water to make a total volume of 50 ml. The mixture was placed in a 250-ml flask

TABLE 1

Percentages of amino acids in identical sardine samples assayed in two laboratories (calculated as per cent of dry weight)

AMINO ACID	FRESH (ACID HYDROLYSATE) <sup>1</sup>				LYOPHILIZED <sup>2</sup>		FROZEN <sup>2</sup>	
	no. 1		no. 2					
	A <sup>3</sup>	B <sup>3</sup>	A	B	A	B	A	B
Arginine	3.1	3.1	3.1	3.1	2.9	3.1	2.9	3.2
Aspartic acid	5.1		5.0		4.7		4.8	
Glutamic acid	7.0		7.0		6.7		7.1	
Glycine	2.9		2.9					
Histidine	3.0	2.9	3.0	3.0	2.8	2.7	2.8	2.8
Isoleucine	2.7	2.7	2.6	2.8	2.5	2.5	2.6	2.5
Leucine	4.1	3.9	4.0	3.9	3.8	3.8	3.9	3.8
Lysine	4.6	4.3	4.6	4.2		4.3	4.5	4.4
Methionine	1.7	1.3	1.6	1.2	1.5	1.5	1.6	1.6
Phenylalanine	2.1	2.1	2.1	2.2	2.0	1.9	2.1	2.0
Threonine	2.4	2.6	2.4	2.6	2.2	2.4	2.3	2.4
Tryptophan	0.5	0.5	0.5	0.5	0.6	0.4	0.6	0.4 <sup>4</sup>
Valine	3.0	3.0	3.0	3.1	2.9	2.7	2.9	2.8

<sup>1</sup> Prepared in the authors' laboratory.

<sup>2</sup> Prepared in the authors' laboratory but hydrolyzed independently in each laboratory.

<sup>3</sup> A — U.C.L.A.

B — University of Wisconsin.

<sup>4</sup> The value 0.5 was found for a hydrolysate prepared according to the procedure of Kuiken et al. ('47).

covered with a beaker, autoclaved for 10 hours at 15 lb. pressure, cooled and filtered. The relatively small amount of residue on the filter paper was washed well with cold 2 N NaOH solution. The filtrate and washings were combined, made up to 100 ml and stored in the refrigerator. Aliquots were neutralized and diluted immediately before making the assays. Tryptophan was the only amino acid determined in

the alkaline hydrolysates. *Lactobacillus arabinosus* 17-5 and essentially the same assay procedure as that described by Greene and Black ('44) were employed.

### RESULTS AND DISCUSSION

Identical or closely agreeing values were found in both laboratories for each of the 10 amino acids determined in the fresh, lyophilized and frozen samples of edible sardine

TABLE 2 .  
*Proximate analysis of canned fish and meat products*<sup>1</sup>

CODE <sup>2</sup>	MOIS- TURE	FAT <sup>3</sup>	ASH	PROTEIN (N × 6.25)	CODE <sup>2</sup>	MOIS- TURE	FAT <sup>3</sup>	ASH	PROTEIN (N × 6.25)
	%	%	%	%		%	%	%	%
MA5P	73.6	2.71	2.75	19.5	SP2P	79.4	0.75	2.29	16.3
MA6P	74.4	3.87	2.69	19.3	SP3P <sup>4</sup>		0.86		14.3
MP3P	70.0	7.70	2.88	21.3	SC3P <sup>4</sup>		23.7		18.6
MP4P	70.1	4.58	2.86	22.2	SC5P	60.1	19.2	1.59	19.1
SO5P	60.5	16.2	3.30	22.3	SH2P <sup>4</sup>		31.0		12.4
SO6P	49.7	18.6	2.54	22.4	SH3P <sup>4</sup>		23.1		15.1
ST4P	64.6	12.1	2.58	19.7	TO2P	49.4	20.4	3.03	24.7
SP1P	77.9	0.63	4.14	16.8	TO4P	56.4	17.2	2.85	24.8

<sup>1</sup> Samples analyzed at U.C.L.A. only. Values for samples analyzed both at U.C.L.A. and the University of Wisconsin are given in table 1 of the second paper (Neilands et al., '49).

<sup>2</sup> A complete explanation of the sample code is given in the text.

<sup>3</sup> The authors are in debt to the University of Wisconsin Alumni Research Foundation for these values.

<sup>4</sup> Sample lost owing to unintentional interruption of refrigeration. Protein was estimated from nitrogen determined in the acid hydrolysate and probably, therefore, is somewhat low due to loss of nitrogen in the humin.

material (table 1). It may be concluded from these results that the microbiological procedures employed were adequate and that the amino acids in fish protein are not altered appreciably by lyophilizing or freezing the fresh material.

The percentages of moisture, ash and protein (N × 6.25) in the samples of fish and meat products analyzed only at U.C.L.A. are given in table 2. The percentages of aspartic acid, glutamic acid and glycine in the proteins of the 41

common samples are given in table 3. The percentages of 13 amino acids determined in the proteins of the fish and meat products assayed only at U.C.L.A. are given in table 4. Although there are quantitative differences between some of

TABLE 3

*Aspartic acid, glutamic acid, and glycine in fish and meat samples<sup>1</sup>*

CODE <sup>2</sup>	ASPARTIC ACID	GLUTAMIC ACID	GLYCINE	CODE <sup>2</sup>	ASPARTIC ACID	GLUTAMIC ACID	GLYCINE
	%	%	%		%	%	%
MA1R	9.2	13.1	5.7	SC1R	9.1	13.2	6.0
MA1P	9.3	13.3	7.0	SC2R	9.3	13.6	6.2
MA2R	9.2	13.2	6.2	SC1P	9.0	13.0	6.4
MA2P	9.0	12.4	6.7	SC2P	8.8	12.6	5.8
MP5R	9.0	12.8	5.6	SH1R	8.7	14.3	6.8
MP5P	8.3	13.0	5.3	SH1P	7.7	13.1	6.1
MP6R	9.0	12.8	5.8	SH6R	8.9	15.8	6.4
MP6P	8.5	12.8	5.4	SH6P	8.5	15.8	6.8
SR <sup>3</sup>	8.7	12.3	5.1	WH1R	9.1	14.8	6.2
SO1R	9.2	13.3	5.6	WH1P	8.6	14.4	6.0
SO2R	7.7	11.2	5.3	WH2R	9.5	15.5	5.9
SO2P	9.2	13.2	6.4	WH2P	8.7	14.6	5.9
ST5R	9.2	13.2	6.6	TO3R	8.3	13.0	4.6
ST5P	8.0	11.3	5.6	TO3RC	8.8	13.6	4.3
ST6R	8.9	11.5	5.2	TO3P	8.4	13.5	4.5
ST6RC	9.6	13.5	5.8	TO5R	8.4	11.4	6.1
ST6P	8.8	12.0	4.9	TO5RC	8.2	11.8	5.0
FA1R	10.2	15.2	5.2	TO5P	8.6	12.2	4.5
FA1P	9.8	14.6	5.0	RB1R	9.2	15.1	5.3
FA2R	9.0	13.6	4.6	RB1P	8.4	14.0	5.3
FA2P	10.1	15.1	5.7				

<sup>1</sup> Values given as per cent protein ( $N \times 6.25$ ). Percentages for 10 other amino acids determined both at U.C.L.A. and the University of Wisconsin are given in the second part of this report (Neilands et al., '49).

<sup>2</sup> A complete explanation of the sample code is given in the text.

<sup>3</sup> A sample of raw Pacific sardines.

the samples, it is of interest that in general there was uniformity of distribution of amino acids similar to that observed previously (Dunn et al., '49a) in the organs and tissues of birds and mammals. It appears that glutamic acid and aspartic acid are major constituents of animal proteins, while

some of the essential amino acids, such as methionine and tryptophan, account for only a small proportion of the weight of any of the animal proteins.<sup>5</sup>

It seems of particular significance that none of the 13 amino acids determined was present in significantly lower

TABLE 4  
*Amino acid content of fish and meat samples*

(Values given as per cent of protein)

CODE <sup>1</sup>	AMINO ACID												
	Arginine	Aspartic acid	Glutamic acid	Glycine	Histidine	Isoleucine	Leucine	Lysine	Methionine	Phenylalanine	Threonine	Tryptophan	Valine
MA5P	5.2	8.2	11.7	7.5	3.4	4.2	7.4	7.8	2.8	3.1	4.6	1.03	5.0
MA6P	5.1	7.4	11.6	6.8	3.2	4.2	7.2	7.6	2.8	3.1	4.7	0.99	4.9
MP3P	5.3	7.9	12.8	5.9	5.6	4.9	7.9	7.8	2.7	3.8	4.1	0.94	5.4
MP4P	4.0	8.1	12.7	5.5	5.7	5.0	7.9	8.0	2.7	3.7	4.3	0.99	5.5
SO5P	5.2	8.1	12.5	5.6	2.0	4.2	6.8	8.3	2.7	3.4	5.1	0.63	4.8
SO6P	5.1	8.4	13.1	4.5	2.1	4.5	7.1	7.9	2.8	3.8	4.2	0.85	5.4
ST4P	5.1	8.8	13.4	5.6	4.7	5.1	7.2	8.7	3.0	3.8	4.5	0.97	5.6
SP1P	8.9	9.7	16.0	5.6	1.84	4.9	8.2	8.1	2.7	4.0	4.1	0.94	4.9
SP2P	8.3	9.3	15.5	6.4	1.72	5.1	8.1	8.0	2.5	3.8	3.9	0.86	4.9
SP3P	8.4	8.9	15.0	7.1	1.68	4.8	7.8	8.2	2.7	3.8	4.3	1.05	5.1
SC3P	5.5	9.0	13.1	6.6	2.4	5.2	7.6	8.5	3.1	3.7	4.3	0.80	5.8
SC5P	5.3	8.5	12.3	6.4	2.4	5.0	7.2	8.1	3.1	3.6	4.3	0.79	5.6
SH2P	6.1	8.6	14.6	6.3	3.0	4.8	7.8	8.7	2.5	3.9	3.2	0.81	5.3
SH3P	5.8	8.2	14.1	6.0	3.0	4.8	7.5	8.2	2.3	3.9	3.1	0.86	4.8
TO2P	5.0	8.7	12.6	4.5	4.9	4.7	7.1	8.2	2.7	3.6	4.2	0.89	5.5
TO4P	5.3	8.6	13.2	4.6	7.1	5.2	7.4	8.6	2.8	3.9	4.7	0.76	5.7

<sup>1</sup> A complete explanation of the sample code is given in the text.

amounts in the canned products than in the corresponding raw samples of fish and meat products. A more complete discussion of the data obtained in both laboratories is given in the second portion of this report (Neilands et al., '49).

<sup>5</sup> Methionine in duck egg albumin appears to be an exception, since it comprises more than 6% of the weight of the protein (unpublished data obtained by Dunn, Shankman and Camien).

## SUMMARY

A study of the amino acid composition of fish and meat products has been made jointly by the authors and by Professors Elvehjem and Strong and collaborators at the University of Wisconsin, with the cooperation of the National Canners' Association and the Can Manufacturers' Institute.

A total of 74 individual samples were assayed in the two laboratories for the 10 essential amino acids. Of this total, 41 samples were assayed in both laboratories and the others were divided into two groups, one assayed only at Wisconsin and the other at U.C.L.A. In addition, aspartic acid, glutamic acid and glycine were determined in all of the samples assayed at the latter institution. It was established that the sample preservation processes and the microbiological procedures employed were adequate. The distribution of amino acids in the canned fish and meat products was generally uniform. None of the 13 amino acids determined was significantly altered by the heat processing to which the canned samples had been subjected. Data from both laboratories are presented and discussed in the second part of this report (Neilands et al., '49).

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# THE NUTRITIVE VALUE OF CANNED FOODS<sup>1</sup>

## II. AMINO ACID CONTENT OF FISH AND MEAT PRODUCTS

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This is the second portion of the report of an inter-laboratory amino acid assay project involving the University of Wisconsin and the University of California at Los Angeles. An introduction to the project, a consideration of the purpose of the work, and a description of the samples analyzed can be found in the immediately preceding paper (part I).

In our laboratory a total of 61 individual samples were assayed for the 10 essential amino acids. Of this total, 41 were identical samples assayed also at U.C.L.A. by Dunn et al. ('49). In the present paper the assay results for the samples assayed in both laboratories will be presented and discussed. Results for the 20 additional samples assayed in this laboratory only will also be presented.

A proximate analysis is given for each sample in order that the amino acid content may be calculated on any desired basis.

<sup>1</sup>Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. The authors are indebted to Miss Martha Johnson for technical assistance with a part of this project. This work was a part of the National Cannery Association—Can Manufacturers' Institute nutrition program, and is No. XXXIII in the series on the nutritive value of canned foods.

## EXPERIMENTAL

*Samples*

Details of the exact nature, manner of collection and distribution of the samples are given in the preceding paper (part I). For convenience each sample was assigned a code designation made up of two initials indicating the type of product, a number to distinguish among multiple samples of the same type of product put up at different canneries and, finally, the letters R, RC or P denoting raw, pre-cooked or heat-processed, respectively. The initial letters used for each type of product were as follows: MA, Atlantic mackerel; MP, Pacific mackerel; SO, Atlantic sardines in oil; ST, Pacific sardines in tomato sauce; FA, Atlantic fish flakes; SP, shrimp; SC, salmon; SH, spiced ham; WH, whole ham; TO, tuna in oil; RB, beef. All the different foodstuffs investigated, with the exception of shrimp, were represented both by processed samples and by a certain number of raw companion samples. The raw samples were received in a frozen state and were held at  $-4^{\circ}\text{C}$ . until used. The heat-processed samples were allowed to remain at room temperature in the original sealed containers.

In almost every instance the entire product, including solid and liquid portions of the canned foods, was homogenized by being passed through a commercial meat grinder several times. The three shrimp samples were handled differently, since only the solid portion of the pack was sampled.<sup>2</sup> Immediately after homogenizing, samples were weighed out for hydrolysis and total N determinations. The product was then placed in a tightly closed screw-cap jar and held in the deep freeze ( $-4^{\circ}\text{C}$ .) until aliquots for dry weight, ash and fat analyses had been taken.

To prepare each sample for tryptophan assay a 5-gm aliquot was placed in a stainless steel beaker and hydrolyzed in 5 N NaOH at  $121^{\circ}\text{C}$ . for 15 hours. This was assumed to race-

<sup>2</sup>At U.C.L.A. both the solid and liquid portions were assayed. These three shrimp samples, therefore, are reported as separate samples assayed at each university (cf. Dunn et al., '49, footnote 3).



mize completely the tryptophan present. The hydrolysate was acidified with excess acetic acid and extracted three times with ether to remove the fat. If glass rather than stainless steel or nickel beakers were used for this hydrolysis a voluminous precipitate, presumably silica, was always obtained on acidification. For the assay of the 9 remaining amino acids the hydrolysate was prepared on a 5-gm aliquot with 3 N HCl at 121°C. for 5 hours.

### *Assay methods*

In our laboratory the proximate analyses (table 1) were carried out as follows: Per cent moisture in the sample was found by drying a 5-gm aliquot to constant weight (usually 48 hrs.) at 100°C., followed by drying overnight over  $\text{CaCl}_2$ . Ash was found by igniting the dry residue from the moisture determination at 800°C. to constant weight (about 6 hrs.). Protein was calculated from total N  $\times$  6.25 where nitrogen was determined by the macro Kjeldahl method. Since the amino acid content of the samples was calculated on the basis of per cent of crude protein, both the Wisconsin and U.C.L.A. protein values are reported for each common sample.

The uniform medium of Henderson and Snell ('48) and the techniques described by these workers were used for all of the amino acid determinations except in analyses where *Leuconostoc citrovorum* 8081 was used as the assay organism. In the latter assays the medium was supplemented with liver concentrate<sup>3</sup> at the level suggested in the recent paper of Steele et al. ('49).

The amino acids used as standards possessed microbiological activity that compared well, within the limits of experimental error, with standards supplied to 20 laboratories for the comparative study of 6 proteins in a project coordinated by Dr. W. H. Cole, Bureau of Biological Research, Rutgers University. A sample of DL-isoleucine supplied by Rutgers University was used as the standard, since other available

<sup>3</sup> Reticulogen, Lilly.

TABLE 1  
*Proximate analysis of fish and meat samples<sup>1</sup>*

CODE <sup>2</sup>	MOISTURE	FAT	ASH	PROTEIN	
				A <sup>3</sup>	B <sup>4</sup>
	%	%	%	%	%
MA1R	65.1	16.3	2.92	17.8	17.4
MA1P	61.7	18.0	2.80	18.0	17.6
MA2R	61.1	19.0	3.01	17.7	17.3
MA2P	61.3	16.7	2.84	18.7	17.8
MA3P	61.7	17.6	3.23	17.7	
MA4P	69.0	5.84	3.59	19.1	
MP1P	68.1	5.47	2.20	26.0	...
MP2P	62.5	8.22	2.19	22.1	.
MP5R	71.5	3.12	2.40	21.4	22.8
MP5P	73.7	3.13	2.83	24.9	20.7
MP6R	73.1	2.36	2.43	20.0	20.7
MP6P	70.5	5.49	2.94	23.1	21.9
SR <sup>4</sup>	70.9	5.08	1.87	20.9	20.6
SO1R	59.5	17.8	2.42	21.3	20.2
SO1P	60.9	15.2	2.42	20.9	
SO2R	68.8	9.95	2.71	21.4	21.3
SO2P	66.8	9.87	2.87	21.8	17.9
SO3P	57.8	17.3	4.52	20.4	
SO4P	53.4	17.8	4.05	23.5	
ST1P	67.0	10.4	2.57	18.6	.
ST2P	63.5	10.3	2.57	18.8	..
ST5R	68.2	10.7	2.87	18.8	17.1
ST5P	67.5	10.4	3.07	17.3	18.0
ST6R	69.9	9.08	2.42	17.5	16.8
ST6RC	67.4	10.5	2.69	19.3	17.1
ST6P	66.5	10.3	2.91	20.5	19.3
FA1R	72.4	0.22	2.16	24.0	25.0
FA1P	68.2	0.31	3.18	24.5	25.3
FA2R	72.9	0.24	1.97	22.8	25.6
FA2P	70.7	0.27	2.45	23.0	25.0
FA3P	68.8	0.43	2.61	24.6	
FA4P	67.3	0.53	2.43	21.0	.
SP1P	69.9	0.63	3.91	26.8	
SP2P	70.1	0.75	2.85	21.4	.
SP3P	72.3	0.86	4.23	21.8	
SC1R	59.0	14.3	2.97	20.2	18.3
SC1P	59.4	17.5	2.24	19.0	18.3
SC2R	65.4	10.4	2.16	20.2	20.4
SC2P	64.7	9.60	2.81	22.7	19.1
SC4P	54.6	24.7	1.41	18.5	.
SC6P	53.6	25.4	1.18	18.5	

TABLE 1 (continued)

CODE <sup>2</sup>	MOISTURE	FAT	ASH	PROTEIN	
				A <sup>3</sup>	B <sup>3</sup>
	%	%	%	%	%
SH1R	54.9	23.8	1.82	14.4	14.8
SH1P	56.0	24.0	2.94	14.6	15.8
SH4P	53.1	24.1	4.39	15.3	
SH5P	54.3	23.7	4.36	15.1	
SH6R	54.0	28.6	3.87	13.1	13.1
SH6P	52.8	26.6	2.40	13.8	13.6
WH1R	60.9	17.5	1.62	15.9	15.4
WH1P	58.8	19.2	2.25	16.0	14.8
WH2R	60.3	19.5	3.61	15.5	16.0
WH2P	56.6	23.9	2.06	17.1	16.5
TO1P	49.2	22.3	2.06	25.6	
TO3R	70.9	0.19	1.53	25.4	25.8
TO3RC	50.4	17.4	2.42	25.6	25.0
TO3P	50.4	23.4	1.53	25.5	25.0
TO5R	63.6	11.8	1.42	25.5	26.6
TO5RC	52.7	18.9	2.82	26.4	29.2
TO5P	51.1	20.9	2.62	25.5	25.6
TO6P	57.2	13.4	3.14	27.7	
RB1R	71.9	4.06	1.21	21.4	20.0
RB1P	68.8	6.88	1.08	22.7	21.5

<sup>1</sup> Values for fat represent the results of a single determination carried out by the Wisconsin Alumni Research Foundation; those for ash are averages of values obtained at Wisconsin and U.C.L.A., while those for moisture are averages of values obtained at Wisconsin, U.C.L.A., and the above Foundation laboratories.

<sup>2</sup> A complete interpretation of the sample code number is given in the text.

<sup>3</sup> A—analyses done at Wisconsin.

B—analyses done at U.C.L.A.

<sup>4</sup> A sample of raw Pacific sardines.

lots of isoleucine appeared to be contaminated, perhaps with alloisoleucine. L-methionine <sup>4</sup> was used for the methionine analyses because previous work in this laboratory had indicated that the D-isomer of DL-methionine had partial activity for *Streptococcus faecalis* R under our assay conditions.

Assays were prepared for simultaneous analyses with two or more organisms wherever this was possible. Accordingly, for each of the amino acids determined at least two of the

<sup>4</sup> We wish to thank Dr. David Doherty for this sample of pure, enzymatically resolved L-methionine.

organisms indicated were used except in the case of lysine and threonine, for which assays only the single organisms are suitable. The different cultures used and the amino acids assayed by each were as follows:

Arginine	<i>L. delbrueckii</i> 3	<i>L. citrovorum</i> 8081
Histidine	<i>L. delbrueckii</i> 3	<i>L. mesenteroides</i> P-60
Isoleucine	<i>L. delbrueckii</i> 3	<i>L. mesenteroides</i> P-60
Leucine	<i>L. delbrueckii</i> 3	<i>L. arabinosus</i> 17-5
Lysine	<i>L. mesenteroides</i> P-60	
Methionine	<i>L. mesenteroides</i> P-60	<i>S. faecalis</i> R
	<i>L. citrovorum</i> 8081	<i>L. fermenti</i> 36
Phenylalanine	<i>L. arabinosus</i> 17-5	<i>L. delbrueckii</i> 3
Threonine	<i>S. faecalis</i> R	
Tryptophan	<i>S. faecalis</i> R	<i>L. arabinosus</i> 17-5
Valine	<i>L. delbrueckii</i> 3	<i>L. arabinosus</i> 17-5

The results were averaged when more than one organism was used for the determination of a given amino acid. This was the case for 380 of the 610 individual values from this laboratory given in table 2.

Titration of the acid formed by the assay organisms was carried out after three days at 37°C. All the analyses done in this laboratory and reported in table 2 were performed on duplicate aliquots of the original sample weighed out separately and hydrolyzed.

To facilitate discussion of the results, table 3 was prepared. It contains an average of the amino acid content of each type of product, both raw and processed, reported in table 2.

#### DISCUSSION

##### *Essential amino acid composition of fish and meat products*

All amino acid values reported and discussed in this paper will be in terms of per cent of crude protein; that is, in grams of amino acid per 16 gm total N.

An inspection of table 3 at once reveals a striking similarity, already noted by Block and Bolling ('45), in the amino acid composition of the various products analyzed. In general, lysine, methionine and tryptophan are the three essential

TABLE 2  
Amino acid content of fish and meat products  
(Values given as per cent of protein, N  $\times$  6.25)

CODE <sup>1</sup>	MA1R		MA1P		MA2R		MA2P		MA3P		MA4P		MP1P		MP2P		MP5R		MP5P		MP6R	
	A <sup>2</sup>	B <sup>2</sup>	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B
Arginine	5.6	5.6	5.8	5.8	5.7	5.7	5.7	5.7	6.1	6.1	5.1	5.1	5.5	5.5	6.1	5.2	5.5	5.4	5.5	5.4	5.4	5.2
Histidine	4.2	4.1	3.7	3.7	3.8	3.9	3.5	3.7	3.7	3.8	5.0	4.8	4.7	4.1	4.7	5.0	4.9	4.6	4.9	5.2	5.6	6.0
Isoleucine	5.5	5.0	5.5	4.8	5.6	5.0	5.6	4.5	5.8	4.7	4.1	4.7	4.1	4.1	4.7	5.0	5.0	6.4	4.3	5.0	4.7	5.0
Leucine	6.9	7.0	7.2	7.4	7.0	7.1	6.9	7.1	7.6	7.5	6.6	7.6	6.6	6.6	7.6	7.8	7.6	7.8	7.1	7.9	6.7	8.1
Lysine	8.0	8.6	7.7	9.0	7.7	8.4	7.6	8.7	8.0	7.4	9.3	7.0	9.6	8.5	8.6	8.0	8.6	8.0	8.6	8.0	8.7	8.3
Methionine	2.6	2.8	2.6	2.8	2.6	2.8	2.5	2.9	2.5	3.2	2.8	2.8	2.8	2.7	2.9	2.8	2.9	2.8	2.9	2.8	2.8	2.8
Phenylalanine	3.3	3.8	3.6	3.6	3.3	3.7	3.4	3.4	3.7	3.3	3.6	3.8	3.6	3.8	4.1	4.0	3.7	3.9	3.7	3.9	3.7	3.9
Threonine	4.6	5.5	4.7	6.3	4.4	4.4	4.4	5.8	4.5	4.0	4.2	5.1	4.4	4.2	4.4	4.2	4.1	4.6	4.1	4.6	4.5	4.6
Tryptophan	0.9	0.9	0.9	0.3 <sup>3</sup>	0.9	0.9	0.9	0.3 <sup>3</sup>	0.9	1.0	1.0	0.9	1.1	1.0	1.1	1.0	1.0	1.0	1.0	1.0	1.1	1.0
Valine	5.6	5.7	5.2	5.4	5.6	5.5	5.4	5.1	5.4	5.2	4.6	5.1	5.5	5.5	5.5	5.5	5.0	5.6	5.0	5.6	4.8	5.6

CODE <sup>1</sup>	MP6P		SR <sup>4</sup>		SO1R		SO1P		SO2R		SO2P		SO3R		SO3P		SO4P		ST1P		ST2P		ST5R		ST5P	
	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B
Arginine	5.6	5.6	4.8	5.0	5.9	5.4	5.6	5.6	6.1	5.5	5.8	4.9	5.3	5.1	4.9	6.0	5.2	4.5	4.9	6.0	5.2	4.5	5.2	4.5	5.2	4.5
Histidine	5.2	5.3	4.9	5.0	2.5	2.6	2.4	2.4	2.3	2.4	2.1	2.2	4.4	4.5	4.4	4.5	5.0	4.3	4.6	5.5	5.0	4.3	5.0	4.3	5.0	4.3
Isoleucine	4.9	5.4	3.6	5.0	5.4	4.8	5.3	5.3	5.3	4.5	5.2	4.1	4.4	4.3	4.4	4.3	4.2	5.2	4.2	5.2	4.5	4.3	4.5	4.3	4.5	4.3
Leucine	6.9	7.4	6.3	6.0	7.2	7.2	7.2	7.2	7.1	6.2	7.3	7.4	7.1	7.1	7.4	7.2	7.1	6.5	8.2	7.3	7.3	7.3	7.3	7.3	7.3	7.3
Lysine	9.1	8.2	8.0	8.5	7.8	8.4	7.8	7.6	7.5	7.5	8.9	7.9	7.0	6.8	8.0	8.6	9.0	9.7	9.7	9.7	9.7	9.7	9.7	9.7	9.7	9.7
Methionine	2.9	2.7	2.5	2.7	2.8	3.0	2.5	2.5	2.6	2.7	3.0	2.5	2.9	3.0	2.8	2.7	2.7	2.9	2.7	2.9	2.4	2.4	2.4	2.4	2.4	2.4
Phenylalanine	3.8	3.9	3.2	3.7	3.5	3.8	3.6	3.6	3.5	3.2	3.5	3.7	3.5	3.7	3.6	3.6	3.7	3.6	3.8	4.2	3.7	3.4	3.7	3.4	3.7	3.4
Threonine	4.3	4.7	3.8	4.2	4.2	3.8	4.2	4.2	4.3	4.8	3.9	5.3	4.2	4.6	4.6	4.6	4.6	4.6	4.3	4.6	4.2	3.6	4.2	3.6	4.2	3.6
Tryptophan	1.2	1.0	1.1	1.1	0.8	0.8	0.8	0.8	0.8	0.7	0.7	0.8	0.7	0.8	0.8	0.9	0.8	0.9	0.9	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Valine	4.7	5.5	4.3	5.4	5.2	5.4	5.4	5.4	5.2	4.2	5.2	4.9	4.7	4.8	4.7	4.8	5.6	5.0	4.8	5.6	5.0	5.3	5.0	5.3	5.0	5.3

TABLE 2 (continued)

CODE <sup>1</sup>	ST6R		ST6RC		ST6P		FA1R		FA1P		FA2R		FA2P		FA3P		FA4P		SP1P		SP2P	
	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B
Arginine	5.0	4.6	5.4	5.4	5.0	4.6	6.1	6.1	6.2	5.8	6.3	5.5	6.2	6.4	6.2	6.4	6.6	6.6	8.6	8.6	10.4	
Histidine	4.5	4.7	4.6	5.1	4.3	4.2	2.1	2.1	2.1	2.1	1.9	1.8	2.0	2.0	2.1	2.0	2.1	2.5	2.1	2.1	2.3	
Isoleucine	4.3	5.4	4.5	5.3	4.3	4.6	6.1	5.2	6.3	4.7	6.1	4.6	6.1	5.2	6.1	5.2	6.1	5.0	5.0	5.8		
Leucine	6.8	7.9	7.3	8.6	7.0	7.5	8.3	8.2	8.2	7.8	8.1	7.2	8.0	8.4	7.9	8.4	7.9	8.4	8.0	9.6		
Lysine	8.9	8.0	9.5	9.4	8.4	7.8	8.5	10.0	8.7	9.4	8.7	8.7	8.4	9.8	8.9	7.3	8.6	8.6	9.0			
Methionine	2.8	2.6	3.0	2.9	2.9	2.8	2.7	3.4	2.6	3.2	3.0	3.0	2.7	3.5	2.7	3.3	3.6	4.0	3.7			
Phenylalanine	3.8	3.6	3.8	4.2	3.7	3.8	3.8	3.9	3.7	3.7	3.8	3.5	3.8	4.1	3.9	3.6	4.0	5.0				
Threonine	4.2	4.6	4.6	5.2	4.0	4.2	4.4	6.0	4.3	5.8	4.5	5.2	4.5	6.0	4.4	4.3	4.3	4.3	4.2			
Tryptophan	1.0	1.0	1.1	1.0	1.1	1.0	1.1	1.1	1.0	1.0	1.0	0.9	1.0	0.9	1.0	1.1	1.1	1.0	1.1			
Valine	4.7	5.7	5.1	6.0	5.1	5.8	5.6	5.4	5.5	5.1	5.3	4.7	5.5	5.4	5.6	5.1	5.1	5.1	5.3			

CODE <sup>1</sup>	SP3P		SO1R		SO1P		SO2R		SO2P		SO6P		SH1R		SH1P		SH4P		SH5P		SH6R	
	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B
Arginine	9.1	5.8	5.5	5.9	5.5	5.7	5.8	5.6	5.4	6.0	6.3	6.2	6.2	6.2	6.3	5.4	6.4	6.4	6.4	6.3	6.4	
Histidine	2.1	2.7	2.7	2.7	2.7	2.6	2.4	2.5	2.3	2.5	2.6	2.8	2.9	2.8	2.7	3.5	3.5	3.5	3.5	2.8	3.3	
Isoleucine	5.1	4.7	5.4	4.9	5.0	4.6	5.2	4.6	4.9	4.8	4.8	4.3	4.5	4.4	4.4	4.7	4.6	4.6	4.4	4.4	4.9	
Leucine	7.9	6.8	7.2	7.2	7.6	7.0	7.5	6.8	7.3	7.5	7.7	7.4	7.6	8.2	6.7	7.9	8.3	7.9	8.3	7.9	8.0	
Lysine	7.9	7.5	8.4	8.0	8.5	7.8	8.8	7.6	8.3	7.5	7.9	8.2	8.9	7.9	7.6	7.7	7.7	7.8	7.7	7.7	8.9	
Methionine	3.3	3.0	2.8	3.1	3.0	3.1	3.0	2.9	2.8	3.5	3.2	2.4	2.4	2.4	2.4	2.0	2.7	2.6	2.6	2.5	2.4	
Phenylalanine	4.4	3.4	3.7	3.7	3.7	3.7	3.8	3.5	3.5	3.6	4.0	3.6	3.9	3.7	3.5	3.6	3.9	3.6	3.9	3.8	4.1	
Threonine	3.9	4.0	4.0	4.6	4.6	4.4	4.4	4.7	4.4	4.3	4.4	4.9	3.6	4.9	2.9	4.6	4.7	4.6	4.7	4.3	4.4	
Tryptophan	0.8	0.9	0.9	1.0	0.8	1.0	0.9	0.9	0.4 <sup>2</sup>	1.0	1.0	0.9	0.9	1.0	0.8	0.9	0.9	0.9	0.9	0.9	0.9	
Valine	5.0	5.3	5.8	5.4	5.9	5.3	5.7	5.2	5.6	5.8	5.6	4.8	5.0	4.9	4.6	4.6	4.9	4.9	4.9	4.9	5.4	

TABLE 2 (continued)

CODE <sup>1</sup>	SHGP		WHGP		WHGP		WHGP		WHGP		TOGP		TOGP		TOGP		TOGP		TOGP	
	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B
Arginine	6.3	6.3	6.1	6.1	6.3	6.2	6.4	6.7	6.9	6.3	5.0	5.3	5.1	5.3	5.0	5.3	5.7	5.2	5.7	5.2
Histidine	3.2	3.2	3.7	3.6	3.4	3.5	3.3	3.6	3.5	3.3	5.8	5.6	6.0	5.8	5.7	6.0	6.0	5.6	6.0	5.6
Isoleucine	4.6	4.9	4.5	5.1	4.7	4.8	4.6	5.3	4.8	5.2	4.1	3.8	4.1	5.0	4.0	4.9	4.8	4.4	4.8	4.4
Leucine	7.7	7.7	7.4	8.1	7.9	7.6	7.6	8.3	8.2	7.8	7.0	6.6	7.0	7.3	6.8	7.3	7.2	6.9	7.2	6.9
Lysine	7.9	8.5	8.2	9.1	9.1	8.8	8.3	9.9	8.7	8.9	8.6	7.7	8.3	8.7	7.6	8.7	8.3	8.6	8.3	8.6
Methionine	2.5	2.3	2.4	2.4	2.5	2.4	2.7	2.8	2.6	2.5	2.7	2.7	2.8	2.7	2.7	2.8	3.0	2.8	3.0	2.8
Phenylalanine	3.5	3.8	3.3	4.1	3.7	4.0	3.5	4.3	3.4	4.0	3.3	3.0	3.5	3.3	3.6	3.1	3.7	3.6	3.4	3.4
Threonine	4.3	4.3	4.1	4.2	4.8	3.5	4.6	3.9	4.7	3.6	3.8	4.0	4.1	4.4	4.7	4.3	4.4	4.5	6.3	6.3
Tryptophan	0.8	0.7	1.0	1.0	0.9	0.8	1.1	1.0	1.1	1.0	1.0	1.1	1.1	1.1	1.0	1.0	0.7	1.0	0.4 <sup>2</sup>	0.4 <sup>2</sup>
Valine	4.8	5.2	5.1	5.5	5.4	5.3	5.2	5.7	5.3	5.4	4.4	4.3	5.2	4.5	5.5	4.3	5.4	5.4	5.4	5.1

CODE <sup>1</sup>	TOGP		TOGP		RBGP		RBGP		RBGP	
	A	B	A	B	A	B	A	B	A	B
Arginine	5.6	4.6	5.6	5.0	5.6	5.8	6.3	6.1	6.0	6.0
Histidine	5.4	5.1	5.6	5.2	6.6	3.8	3.7	3.6	3.4	3.4
Isoleucine	5.1	4.8	5.3	5.4	5.3	4.9	5.5	4.7	5.0	5.0
Leucine	7.2	7.2	7.5	7.2	7.9	7.6	8.3	7.4	7.7	7.7
Lysine	8.0	8.1	8.5	8.1	8.3	8.1	9.7	8.0	8.8	8.8
Methionine	2.9	2.7	2.9	2.6	3.1	2.7	2.7	2.7	2.5	2.5
Phenylalanine	3.8	3.3	4.0	3.7	4.1	3.8	4.1	3.4	3.7	3.7
Threonine	4.7	4.5	4.7	4.5	4.0	4.4	4.9	4.5	4.3	4.3
Tryptophan	1.0	0.4 <sup>2</sup>	1.0	0.9	1.0	1.0	1.1	0.9	0.9	0.9
Valine	5.5	5.2	5.6	5.7	5.8	5.2	5.5	4.9	5.0	5.0

<sup>1</sup> A complete explanation of the sample code is given in the text.<sup>2</sup> A — assays done at Wisconsin.

B — assays done at U.C.L.A.

<sup>3</sup> These values appear to be out of line. The reason for this is unknown.<sup>4</sup> A sample of raw Pacific sardines.

TABLE 3  
Average amino acid content of fish and meat samples<sup>1</sup>  
(Values given as per cent of protein, N  $\times$  6.25)

NO. OF SAMPLES	ATLANTIC MACKEREL	PACIFIC MACKEREL	ATLANTIC SARDINES	PACIFIC SARDINES	FISH FLAKES	SHRIMP	SALMON	SPICED HAM	WHOLE HAM	TUNA	BEEF
	6	6	6	8	6	3	6	6	4	8	2
<i>Amino acid</i>											
Arginine	5.8	5.5	5.5	5.1	6.1	9.4	5.8	6.2	6.4	5.3	6.1
Histidine	3.8	5.4	2.4	4.7	2.1	2.2	2.6	3.1	3.5	5.7	3.6
Isoleucine	5.2	5.0	4.9	4.6	5.5	5.3	4.9	4.6	4.9	4.7	5.0
Leucine	7.2	7.4	7.1	7.2	8.1	8.5	7.3	7.7	7.9	7.2	7.8
Lysine	8.1	8.5	7.8	8.4	8.8	8.5	8.0	8.1	8.9	8.3	8.7
Methionine	2.7	2.8	2.7	2.8	3.0	3.4	3.0	2.4	2.5	2.8	2.7
Phenylalanine	3.5	3.8	3.4	3.7	3.9	4.5	3.7	3.8	3.8	3.5	3.8
Threonine	4.9	4.5	4.4	4.3	4.9	4.1	4.4	4.3	4.2	4.5	4.5
Tryptophan	1.0	1.0	0.8	1.0	1.0	1.0	0.9	0.9	1.0	1.0	1.0
Valine	5.4	5.2	5.0	5.2	5.3	5.1	5.6	4.9	5.4	5.1	5.2

<sup>1</sup> Average of all values in table 2 except those marked and indicated to be out of line.



amino acids most important in evaluating the nutritive quality of a protein. A deficiency of these amino acids is frequently encountered in natural food materials. All the products tested in this survey are apparently very good sources of lysine and good sources of tryptophan and methionine and the other essential amino acids. These products must be considered to contain protein of the finest nutritive quality.

In marked contrast to the very small variation found for each specific amino acid in *different* products, we record one exception, i.e., histidine. Commenting upon a similar observation, Lyman states in a recent publication (Lyman and Kuiken, '49) "... variation in the histidine content of muscle tissue is due to the presence of substantial amounts of derivatives of histidine which are not part of the true protein of the tissue. These histidine derivatives are carnosine, a peptide of beta-alanine and histidine, and anserine, a peptide of beta-alanine and methyl histidine. A study of the relationship of these compounds to the histidine content of meat has been reported by Lyman, Kuiken and Hale ('47). As much as one-third of the total histidine content of beef loin or other beef muscle tissue may be in the form of carnosine."

The existing literature contains relatively little information on the amino acid content of the edible portions of various fish, and even where data are available comparisons are often difficult because the fish analyzed have been inadequately identified. In the following discussion the methods used for determining the amino acids in question are indicated in parentheses. Further details regarding the indicated methods may be found in Block and Bolling ('45).

Pottinger and Baldwin ('39) have investigated the arginine, histidine, lysine (Block) and tryptophan (Folin and Ciocalteu) content of a series of fishery products. Although only 4 amino acids were determined, the materials analyzed were identified by their scientific names and therefore we believe that our results can be compared with confidence to those of Pottinger and Baldwin.

Atlantic mackerel assayed by these workers was found to contain 5.8% arginine, 1.9% histidine, and 7.1% lysine. Our analysis of this species (table 3) shows 5.8% arginine, 3.8% histidine and 8.1% lysine. Pottinger and Baldwin mention that since their methods depend on the isolation of the amino acids as a salt, their results must be regarded as minimum. The tryptophan content was found by them to be 1.4% as compared to 0.98% in our analysis.

The same authors found that mature Atlantic herring contained 5.1% arginine, 1.6% histidine, 7.0% lysine and 1.2% tryptophan, as contrasted to 5.5, 2.4, 7.8 and 0.77%, respectively, for our samples.

Pilchards (*Sardinia pilchardii*) contained 5.6% arginine, 1.2% histidine, 6.8% lysine and 1.3% tryptophan, while our Pacific sardines (*Sardinia caerulea*), probably a very closely related species, contained 5.1% arginine, 4.7% histidine, 8.5% lysine and 0.98% tryptophan.

Cod and haddock analyzed by Pottinger and Baldwin and the Atlantic fish flakes assayed by the present authors contained, respectively, 5.6, 5.7 and 6.1% arginine; 1.7, 1.2 and 2.1% histidine; 6.8, 6.4 and 8.8% lysine; and 1.06, 0.85 and 0.99% tryptophan.

It is noteworthy that the products assayed by chemical methods as contrasted with the newer microbiological assays were consistently very similar for arginine content, somewhat lower for lysine, higher for tryptophan and much lower for histidine. Fuller et al. ('47) believe that the carnosine and hence the histidine content of muscle tissue may vary with the amount of dietary histidine.

Cod, analyzed by Beach et al. ('43), and the Atlantic fish flakes of this survey contained, respectively, 6.3% (Block) and 6.1% arginine; 2.0% (Block) and 2.1% histidine; 8.4% (Block) and 8.8% lysine; 3.7% (Sullivan) and 3.0% methionine; 4.3% (Block) and 3.9% phenylalanine and 1.3% (Lugg) and 0.99% tryptophan. Again the microbiological method gave a lower tryptophan content. The other analyses are in fairly good agreement.

Edwards et al. ('46) determined the essential amino acids in fresh haddock. They found 9.2% arginine (Weber-Bartlett), compared to 6.1% arginine for our Atlantic fish flakes. The values for phenylalanine (Block) and tryptophan (Lugg, Brand and Kassell) were very close to those found by the present authors using microbiological methods.

Shrimp has been rather thoroughly assayed for amino acid content. Beach et al. ('43) obtained remarkably similar figures to those in table 3 for histidine (Block), lysine (Block), methionine (Sullivan), phenylalanine (Block) and tryptophan (Lugg). They found an arginine content of 6.6% (Block), compared to 9.4% in our samples. The shrimp sample analyzed by Pottinger and Baldwin ('39) contained 7.5% arginine (Block) and was considerably higher in this amino acid than the true fish products assayed. Possibly the phosphoarginine (Baldwin, '47) of invertebrate muscle may account for the higher arginine values found in these instances.

Employing chemical methods to examine salmon for 5 of the essential amino acids, Beach et al. ('43) obtained results in fair agreement with those reported for salmon in table 3. Deas and Tarr ('49) have investigated the amino acid content of various fishery by-products using the microbiological assay.

In contrast to the scarcity of data for the amino acid composition of fish, the literature affords relatively ample data for various types of meat samples. Table 4 was drawn up to compare our values for pork and meat with those in the literature. This table again illustrates the similarity of the essential amino acid composition of animal proteins. Differences between our averages and those in the literature are probably caused primarily by inherent fluctuations in the assay method, plus the effect of abnormally high or low figures on the literature average.

#### *Reliability of the microbiological assay*

In reviewing the literature of amino acid analysis the authors were impressed by the oft-repeated statement that the chemical determination of only a few of the essential

amino acids in a relatively small number of products could develop into a formidable task. The microbiological assay eliminates much of the tedious work required by chemical methods, and renders more extensive surveys feasible. However, the newness of the microbiological methods makes more data on their reliability highly desirable, and the present study provides some information on this question.

TABLE 4

*Comparison of the present data with values in the literature for the essential amino acid composition of pork and beef*

(Values given as per cent of protein,  $N \times 6.25$ )

AMINO ACID	PORK MUSCLE				BEEF MUSCLE			
	Present <sup>1</sup> project	Literature <sup>2</sup>			Present <sup>1</sup> project	Literature <sup>2</sup>		
		Ave.	Range	No. of analyses		Ave.	Range	No. of analyses
Arginine	6.3	6.5	6.4-6.6	2	6.1	7.0	5.7-10.1	9
Histidine	3.3	3.0	2.2-3.8	2	3.6	2.5	0.6-4.4	9
Isoleucine	4.8	5.5	5.1-5.9	2	5.0	5.5	5.2-5.9	4
Leucine	7.8	8.0	7.2-8.6	2	7.8	7.9	7.5-8.6	4
Lysine	8.5	8.7	8.7-8.7	2	8.7	8.2	7.3-10.0	9
Methionine	2.5	2.9	2.4-3.4	3	2.7	3.3	2.5-4.1	5
Phenylalanine	3.8	4.0	3.7-4.2	3	3.8	4.2	3.6-4.9	6
Threonine	4.3	4.6	4.5-4.8	3	4.5	4.6	3.5-5.3	5
Tryptophan	0.9	1.3	1.2-1.4	4	1.0	1.3	0.9-1.5	11
Valine	5.2	5.4	5.3-5.4	2	5.2	5.2	5.1-5.3	4

<sup>1</sup> This column was taken from table 3 and is, therefore, the average of all samples of each product.

<sup>2</sup> Literature values are from the paper of Lyman and Kuiken ('49).

The inter-laboratory agreement on results obtained from the 41 common samples assayed for 10 amino acids can be seen in table 2. To express the degree of variation among these results all values for a given amino acid were averaged, and the standard deviation between the two laboratories expressed as a percentage of this mean. The coefficients of variation so calculated ranged from 5.2% for histidine to 17.5% for tryptophan, with an average for all amino acids of 8.9%. It will be noted that different organisms were used

in the two laboratories to determine arginine, histidine, isoleucine, methionine, phenylalanine and threonine.

Similarly, the variation among replicate determinations made with a given test organism in this laboratory was found to range from 3.5% for leucine assayed with *L. delbrueckii* to 12.0% for methionine assayed with *L. fermenti*, with an average for all amino acids and all test organisms of 8.0%.

The results obtained in this laboratory when two organisms were used to determine the same amino acid in a series of samples were also compared. The variation between organisms was significantly greater than that between replicates obtained with the same organism in 4 out of the 10 cases for which data permitting such comparisons were available, namely, leucine, valine, and phenylalanine assayed with *L. arabinosus* and *L. delbrueckii*, and methionine assayed with *L. mesenteroides* and *S. faecalis*. However, the actual amino acid values obtained with different organisms showed a statistically significant difference in only two cases. The average phenylalanine content of 30 samples was found to be 3.45% (of the protein present) with *L. arabinosus* and 3.95% with *L. delbrueckii*. Similarly, the average valine content of 32 samples was 5.29% with *L. arabinosus* and 4.71% with *L. delbrueckii*.

It is felt that these comparisons provide strong evidence of the reliability of microbiological methods in determining the 10 amino acids studied.

### *The effect of heat processing*

The data for raw and heat-processed samples in table 2 show no significant effect of heat processing on the essential amino acid composition of the samples analyzed.

### SUMMARY

An inter-laboratory amino acid assay project has been described. A total of 61 raw and processed samples of fish and meat were assayed, of which 41 were analyzed at both the University of California at Los Angeles and the University of Wisconsin.

The essential amino acid composition of the various species of fish investigated was found to be in fair agreement with that found by earlier workers, although some differences are noted and discussed. Excellent agreement was found with respect to the 41 samples which were assayed in both laboratories. This comparison gives evidence of the reliability of the microbiological assay technique for amino acid analysis. Heat processing as employed in canning did not change materially the essential amino acid composition of the various fish and meat samples assayed.

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# THE INFLUENCE OF OPTICAL ISOMERISM AND ACETYLATION UPON THE AVAILABILITY OF TRYPTOPHAN FOR MAINTENANCE IN MAN<sup>1</sup>

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FIVE FIGURES

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Tryptophan is essential for the maintenance of nitrogen balance in man (Holt and others, '41; Rose, '47), hence must be present to make any protein or mixture of amino acids nutritionally complete. Since it is destroyed by the conditions usually employed in the acid hydrolysis of proteins, it must be added to acid hydrolysates designed for emergency use as protein substitutes. At present, the natural L form of tryptophan can be obtained commercially in limited quantities, but its cost is prohibitive in comparison with that of the synthetic DL modification. These circumstances obviously make it desirable to know how well the needs of the human subject for the L form can be met by supplying the DL mixture. It is with the comparative availabilities to man of the D and L isomers of tryptophan that this communication is chiefly concerned.

<sup>1</sup> The experimental data in this paper are taken from a dissertation submitted by Heber R. Baldwin in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biochemistry in the Graduate College of the State University of Iowa in February, 1949. These studies were made possible by a generous grant-in-aid from the Dow Chemical Company of Midland, Michigan, to whom we are also indebted for the supply of DL-tryptophan used.

Since this work was begun, about 30 months ago, Albanese, Davis and Lein ('48) have reported that D-tryptophan is unable to support nitrogen balance when it is fed to normal male infants as a component of DL-tryptophan, but that it becomes as effective as L-tryptophan when the racemic mixture is acetylated. This is quite the reverse of the situation which exists in the rat. In this species, D-tryptophan is utilized for growth almost as well as L-tryptophan; acetylation does not impair the availability of the L isomer but completely prevents the use of the D form for this purpose (du Vigneaud and others, '32; Berg, '34).

In support of their assertion that acetylation makes D-tryptophan available to the human infant for tissue synthesis, Albanese, Davis and Lein ('48) cite other data which seem too remotely related to be significant, involving as they do: (a) the excretion in the urine of an "aberrant" metabolite following the administration of 0.01 mol (about 2 gm) of DL-tryptophan to the normal human adult after an overnight fast (Albanese and Frankston, '44) and (b) failure to find the same unknown metabolite when acetyl-DL-tryptophan is fed, or to account for as much of the original 0.01 mol of acetylated derivative as when acetyl-L-tryptophan is given (Albanese, Frankston and Irby, '45; Albanese, Davis and Lein, '48). To infer that inability to account for a product fed indicates that it has therefore been converted into an available metabolite is quite unwarranted. In the instance cited, rupture of the indole ring would alone have sufficed to convert the acetyl-D-tryptophan into an available product; one which, moreover, might not be detectable by the method of assay employed. It is also important to note that mechanisms called into play to dispose of a relatively large excess of an amino acid supplied singly may differ markedly from those involved in the utilization of the relatively small quantity that need be supplied in the diet for maintenance and growth. This is certainly true of both free and acetylated D- and L-tryptophan in the rat, strongly probable in the rabbit and the



dog, and therefore a possibility that cannot safely be ignored in man.

The studies summarized in the present paper involve the responses of normal male adult subjects, as measured by nitrogen balance, to diets containing acid-hydrolyzed casein, with or without supplements of free or acetylated tryptophan, as the source of protein nitrogen. To make direct isomeric comparisons possible, free and acetylated D- and L-tryptophan

TABLE 1

*Physical properties and nitrogen content of the free and acetylated isomers of tryptophan*

COMPOUND	m.p. (uncorr.) <sup>1</sup>	[ $\alpha$ ] <sub>D</sub> <sup>20</sup> <sup>2</sup>		TOTAL NITROGEN <sup>3</sup>	
		Found	Recorded (Berg, '33)	Found	Theory
	°C.	degrees	degrees	%	%
L-Tryptophan	278-280	- 32.0	- 32.1	13.69	13.72
D-Tryptophan	278-280	+ 31.6	+ 32.45	13.66	13.72
DL-Tryptophan	279-282	0.0		13.65	13.72
Acetyl-L-tryptophan	187	+ 26.0	+ 26.1	11.30	11.38
Acetyl-D-tryptophan	186	- 25.2	- 26.2	11.28	11.38

<sup>1</sup> The samples were heated in open capillary tubes. The temperature of the oil bath was raised 3°C. per minute.

<sup>2</sup> The aqueous solutions of tryptophan contained 0.5 gm per 100 ml of solution; the methanol solutions of acetyl-tryptophan, 1 gm per 100 ml of solution.

<sup>3</sup> Determined by the macro-Kjeldahl method.

were prepared by resolution for use along with the free DL mixture. So far as we are aware, data involving direct comparisons have not previously been published. A résumé of studies including similar tests has, however, very recently been presented by Rose ('49). With his findings our data are in essential agreement.

#### EXPERIMENTAL

Data pertaining to the free and acetylated L- and D-tryptophan used in these tests are presented in table 1. These compounds were prepared from synthetic DL-tryptophan essentially as directed by Berg ('33).

*Tests with diets containing items low in nitrogen and tryptophan as sources of the non-protein requisites*

Serious efforts were made initially to devise a tryptophan-deficient diet in which an acid hydrolysate of casein<sup>2</sup> constituted the chief source of nitrogen, but the additional caloric requirements and the mineral and vitamin needs were to be met chiefly by commonly used dietary items, such as fruits, vegetables, fats, sugars and starches, with as little nitrogen or tryptophan as possible.<sup>3</sup> This approach had been used by Holt and his associates ('41) and was adopted in the hope that a palatable, somewhat varied, but fairly natural tryptophan-low diet could thus be provided. In the initial tests, removal of the daily supplement of tryptophan (250 mg) which had been fed during the 12 days allowed for adjustment to the new diet failed to produce a definitely negative nitrogen balance even after 12 days or more had elapsed. Therefore, to afford a more certain basis for selection, representative samples of the various items of the diet were homogenized, hydrolyzed with barium hydroxide and assayed for tryptophan by the microbiological method of Greene and Black ('44), in which lactic acid production by *Lactobacillus arabinosus* 17-5 is measured. The information thus acquired made it possible to detect and eliminate several unsuspected dietary items with a relatively high content of tryptophan. Assay of aliquots of the mixed diets indicated that they provided 135 mg of tryptophan per day. More rigid selection allowed a reduction in the tryptophan content to 115 mg per day, but the revised diet again failed to produce a distinctly negative balance when the initial supplements of tryptophan were removed. Since still further elimination of tryptophan

<sup>2</sup>We are grateful for the assistance of Dr. Charles F. Kade, Jr., and the Sterling-Winthrop Research Institute, Rensselaer, New York, and of Dr. John T. Correll and Dr. Curtis E. Meyer and the Upjohn Company, Kalamazoo, Michigan, who supplied us with sizable lots of acid-hydrolyzed casein.

<sup>3</sup>The advice of Dr. Kate Daum and Miss Evelyn Brandt of the Nutrition Department of the University Hospitals and their cooperation in making the facilities of the diet kitchen available to us in this phase of the study are gratefully acknowledged.

was impractical, this type of diet was abandoned in favor of one that was highly synthetic.

Although the preliminary studies outlined above failed to meet our needs, they did serve to emphasize that the minimum daily requirement of tryptophan is certainly low and that no appreciable deficiency is likely to be produced by the ordinary type of diet if enough is ingested to meet caloric needs.

### *Tests with synthetic diets*

The dietary regimen finally adopted was patterned after the type so successfully and extensively employed by Rose ('47). Table 2 provides information as to basic composition. In making the wafers, the dry ingredients were thoroughly mixed and the butter and commercial hydrogenated cotton-seed oil<sup>4</sup> melted and stirred in. Approximately 4 l of water were then added, the batch was transferred to a Hobart mixer and stirred thoroughly for several minutes, and the batter was spread in small pools on cookie sheets and baked at 375°F. for 20 to 25 minutes.<sup>5</sup> The baked product was golden brown, almost paper-thin, crisp and tasty. For convenience, it was packaged in 100-gm lots and refrigerated until used. From the initial 7,670 gm of ingredients per batch, 6,500 to 6,800 gm of wafers were obtained. The amount of Cellu flour which they provided was usually sufficient to prevent the extremes of diarrhea and constipation. In a few instances, 5 to 10 gm more were supplied daily in water suspension.

Analyses of wafers from the various bakings showed that, per 100 gm, they contained approximately 52 mg of total N (by macro-Kjeldahl) and 1.5 mg of tryptophan (by the method of Greene and Black, '44). Values calculated from the analyses of the various individual items agreed well with those obtained by direct analyses of the mixtures. Components other than the acid-hydrolyzed casein and the supplements of free

<sup>4</sup> Crisco.

<sup>5</sup> We are greatly indebted to Dr. Sybil Woodruff and Miss McDivitt of the Home Economics Department for their counsel and the use of their equipment and kitchen facilities for mixing and baking these wafers.

TABLE 2  
*Composition of the basal diet*

DIETARY COMPONENT	DAILY ALLOTMENT	INGREDIENTS USED PER BATCH OF WAFERS	VITAMINS PER CAPSULE
Acid-hydrolysate of casein	0.1 gm of N/kg of body wt.	Corn starch	A
		Sucrose	D
		Butter	Ascorbic acid
		Crisco	Thiamine HCl
Wafers	400-500 gm	Mineral mixture	Riboflavin
Butter	50 gm	(Murlin and others, '46)	Nicotinamide
Sucrose	200 gm	Sodium chloride	Pyridoxine HCl
Lemon juice	100 ml	Baking powder <sup>2</sup>	Ca pantothenate
Coffee	300 ml	Cellu flour <sup>3</sup>	
Vitamins <sup>1</sup>			
Upjohn Unicap	1 capsule		
Folic acid	5 mg		
		470	
		7,670	

<sup>1</sup> We are indebted to Dr. John T. Correll and the Upjohn Company, Kalamazoo, Michigan, for our supply of these vitamins.

<sup>2</sup> Composed of  $\text{NaHCO}_3$ ,  $\text{Ca}(\text{H}_2\text{PO}_4)_2 \cdot \text{H}_2\text{O}$ , and corn starch in the ratio of 22:29:27 gm.

<sup>3</sup> Type B, Chicago Dietetic Supply House, Inc., Chicago, Illinois.

or acetylated tryptophan furnished less than 0.45 gm of total nitrogen per day, and together with the daily allotment of tryptophan-deficient hydrolysate, less than 25 mg of tryptophan.

Different lots of acid-hydrolyzed casein varied somewhat in taste and to a minor degree in nitrogen content, hence possibly also in amino acid composition. Each subject was therefore fed the same lot of hydrolysate throughout the experimental period. The daily allotment was weighed out, mixed with the tryptophan supplement when this was provided and dissolved in water. A third of this solution was consumed during each of the three meals. Enough wafers were eaten to raise the daily caloric intake to 42 Cal. or more per kilogram of body weight. In calculating caloric content, sucrose and starch were assumed to provide 4.0 Cal. each per gram, butter 8.0, the casein hydrolysate 4.0, and the wafers 4.3. Preliminary tests showed that in the subjects tested on this regimen, supplements of 200 mg of L-tryptophan per day were sufficient to establish nitrogen equilibrium.

Six normal male adults served as the experimental subjects in the final tests.<sup>6</sup> Of these, two were forced to discontinue the diet prematurely because of gastrointestinal distress characterized by nausea, anorexia, diarrhea and vomiting. The others had shown mild anorexia and nausea on the deficient diet, but not when tryptophan was provided.

Urine samples were collected from 8:00 A.M. to 8:00 A.M. on successive days. Each sample was analyzed for total nitrogen by the macro-Kjeldahl method. Creatinine N was determined routinely as a check on the collection of urines. Stools were collected daily and assayed for total nitrogen. To insure correspondence of fecal collections with the periods on the different diets, carmine was ingested as a marker each time the diet was changed. Average daily fecal nitrogen output per period was used in the calculations of nitrogen balance.

<sup>6</sup>To these and the several other experimental subjects who cooperated splendidly, we express sincere appreciation.

During the period of adjustment to the synthetic regimen, the basal diet was supplemented with 200 mg of L-tryptophan per day. From three to 14 days were required to attain nitrogen equilibrium, and 5 days more were usually allowed. Preliminary trials had shown that after this adjustment period responses to the omission or addition of tryptophan occurred promptly, hence that a period of 4 to 6 days was long enough to indicate whether a supplement could or could not be utilized to prevent or correct a negative nitrogen balance.

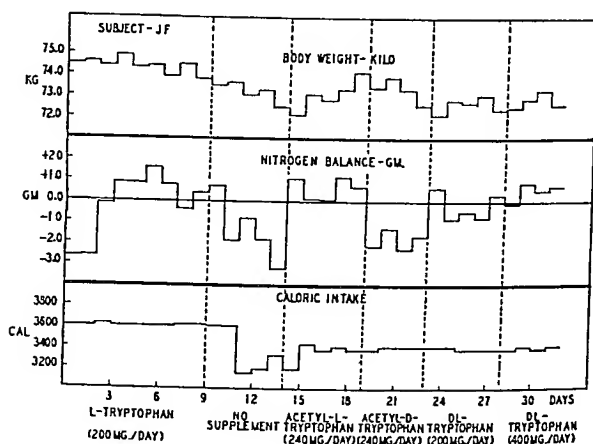


Figure 1

The data obtained on body weight, nitrogen balance and caloric intake in 5 of the 6 experimental subjects are compiled and presented in figures 1-5. Data on the 6th subject are omitted since he was forced to discontinue the diet during the initial period of adjustment.

#### DISCUSSION OF RESULTS

Subject J F (fig. 1) maintained a fairly even caloric intake during his 32 days on experiment. Withdrawal of tryptophan produced a negative nitrogen balance. The accompanying drop in caloric intake to 43 Cal. per kilogram reflected the anorexia and nausea experienced during this period. The lowered caloric intake would probably have sufficed to main-

tain nitrogen equilibrium had the diet contained enough tryptophan. In this subject, 240 mg of acetyl-L-tryptophan (molecularly equivalent to 200 mg of free tryptophan) promoted a positive balance, but a similar amount of acetyl-D-tryptophan was without effect. A daily intake of 200 mg of DL-tryptophan failed to insure equilibrium. The response on 400 mg of DL-tryptophan was similar to that on 200 mg of the L-form or 240 mg of the acetyl-L-derivative. In general, the changes in body weight seemed to parallel approximately the changes in nitrogen balance.

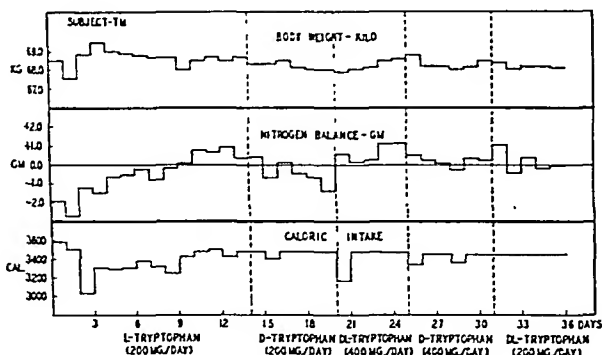


Figure 2

With few exceptions, subject TM (fig. 2) maintained an intake of 50 Cal. per kilogram per day throughout his 36 days on the study. As in the previous subject, 400 mg of DL-tryptophan were adequate for maintenance but 200 mg were not. The response to 200 mg of the D isomer was definitely negative, but to 400 mg it was only slightly inferior to that obtained on 200 mg of L-tryptophan. Changes in body weight were less marked in this subject, but again were roughly parallel to the changes in nitrogen balance.

The results obtained on WH (fig. 3) were not very satisfactory. On the 17th day this subject complained of gastrointestinal distress, nausea and anorexia. His stools were loose. The condition could not be cleared up, and the study was terminated on the 25th day by a siege of vomiting. This

subject's negative nitrogen balance during the period on *D*-tryptophan was undoubtedly attributable in part to the lowered caloric intake which began at the onset of his symptoms of distress.

The data obtained on subject L G (fig. 4) again indicate the inadequacy of a supplement of 200 mg of *D*-tryptophan per

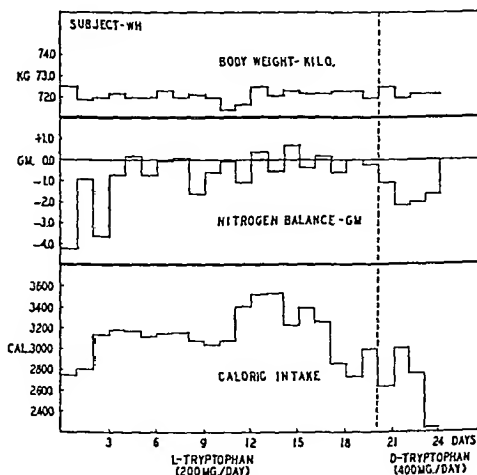


Figure 3

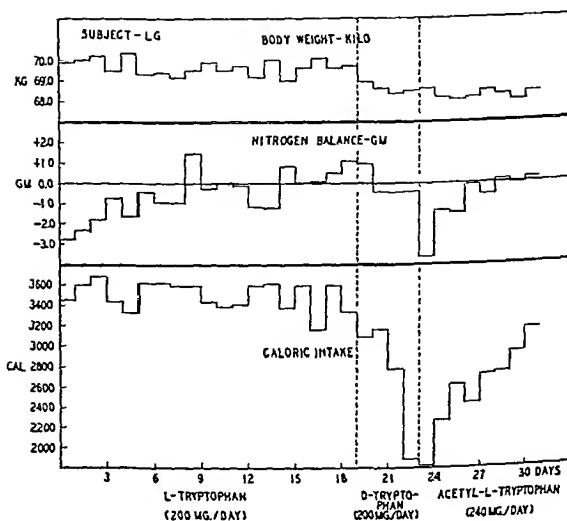


Figure 4



day. During the period on this supplement the subject experienced extreme lassitude, anorexia and nausea and could not be induced to maintain his previous caloric intake. Replacement of the D-tryptophan by an equimolar supplement of acetyl-L-tryptophan brought about a gradual return of appetite, an increased caloric intake and ultimately a restoration of nitrogen equilibrium.

The results obtained with subject R S (fig. 5) indicate again that acetyl-L-tryptophan is an effective substitute for

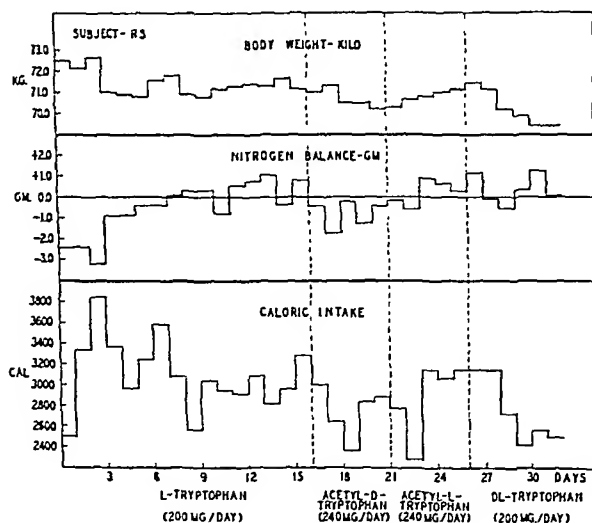


Figure 5

L-tryptophan and that 200 mg of DL-tryptophan are borderline or suboptimum in their effects. As in subject J F (fig. 1), the administration of 240 mg of acetyl-D-tryptophan per day led to a pronounced loss of appetite, an increasing nausea, and a negative nitrogen balance.

The subjective responses of the men to the various regimens were of interest, especially since none of them was informed of what to expect, either in advance of or during the change in type of diet or in level of supplementation. With a single exception (W H), none of the individuals was trained in biochemistry or nutrition or had any knowledge of symptoms

that had been encountered by others in similar series of tests. It is unlikely, therefore, that their reports of depressed appetite, nausea, increased nervous irritability, and so forth, were attributable to suggestion.

The difficulties encountered in attempting to maintain constant and adequate caloric intakes, especially during a period of dietary deficiency, are quite in line with observations made repeatedly on experimental animals. Similar symptoms have been reported by Rose ('47) in the human subject during periods of negative nitrogen balance.

At best, casein hydrolysates are far from tasty. Such untoward responses to their oral use as loss of appetite, nausea and decreased food intake have been noted (e.g., Co Tui, '46). Our 15 subjects, after the first few days, became accustomed, though perhaps never fully reconciled, to their strong taste. Several attempts were made to mask the objectionable flavor but these were ultimately abandoned in favor of the procedure of dissolving the hydrolysates in water, the subject drinking the solution and following this with a chaser of lemonade.

Holt and associates ('41) report that it sometimes required several days after the removal of the tryptophan supplement for subjects on their diets to go into negative nitrogen balance, also as long as a week after its replacement to restore nitrogen equilibrium. The rapid responses of our subjects to such changes can probably be attributed to a more rigorous exclusion of tryptophan from their basal diets and to their higher caloric intakes.

The studies reported in this paper were not designed to determine the amount of tryptophan needed to maintain nitrogen balance in the human adult, but the data recorded in figures 1 to 5, together with those from preliminary studies on 10 other subjects, indicate that nitrogen equilibrium can usually be maintained or restored on 225 mg of L-tryptophan per day and, in some instances, apparently on even less. In any event, the amount needed is certainly far less than the 6.0 to 9.0 mg per kilogram per day (420 to 630 mg for a 70-kg

subject) set by Holt and others ('44). Our data favor the minimum estimates of 250 mg to 150 mg per day suggested by Rose ('47, '49).

As indicated earlier, the deductions of Albanese, Davis and Lein ('48) concerning the availability of the *D* isomers in the male infant were based on responses to the free and acetylated *DL* mixtures. In general, our tests seem to indicate that *DL*-tryptophan is much less well utilized for maintenance than is *L*-tryptophan. The tests with *D*-tryptophan suggest that this component is certainly very poorly utilized, but leave some doubt that no use at all can be made of it, if the caloric intake is high and the amount ingested is sufficiently large. Our findings on acetyl-*D*-tryptophan do not agree with the conclusion of Albanese, Davis and Lein ('48) that the *D*-component of acetyl-*DL*-tryptophan can be utilized to maintain nitrogen balance. Their allowance of acetyl-*DL*-tryptophan was 48 mg per kilogram per day (259 to 356 mg per subject per day). The presence of the utilizable *L* isomer renders it more difficult to assess with certainty the effect of the *D* isomer alone. This would be particularly true if the amount fed appreciably exceeded the required minimum. At least in experimental animals, and presumably also in human subjects, borderline deficiencies are accompanied by variations in response which are wider and hence more difficult to evaluate than are the narrower differences noted when deficiencies are marked.

### *Excretion of tryptophan*

The apparent urinary excretion of free *L*-tryptophan, as measured by the Greene and Black ('44) microbiological method of analysis, without autoclaving with barium hydroxide, averaged 5.0 to 9.1 mg per day on the various supplemented regimens. After autoclaving the urine for 16 hours at 15 lb. pressure with 5 N barium hydroxide (and doubling the value obtained, to compensate for the fact that the test organism responds only to the *L*-component of the resulting racemic mixture), the average responses on the various regimens in-

licated the following apparent outputs of free and combined tryptophan: on daily supplements of 200 mg of L-, DL- and D-tryptophan, respectively, 27.1, 38.5 and 72.6 mg per day; on daily supplements of 400 mg of DL- and D-tryptophan, 65.6 and 126.8 mg, respectively, per day; and on daily supplements of acetyl-L- and acetyl-D-tryptophan equivalent to 200 mg of tryptophan, 24.7 and 69.8 mg per day. To judge from the low values obtained with test solutions of acetyl-tryptophan, the last two figures may represent only half to three-fourths of the total excretion. It is therefore also possible that tryptophan bound in any other form difficult to hydrolyze may not have responded completely to the test; on the other hand, it is also possible that certain related intermediates or catabolites, or substances produced by the autoclaving, may respond and hence be assayed as tryptophan. Tests indicated that free D-tryptophan would beyond doubt have been completely racemized during the autoclaving with the barium hydroxide, hence would have been measured.

#### SUMMARY AND CONCLUSIONS

Comparative tests have been made of the relative capacities of free and acetylated L- and D-tryptophan and of DL-tryptophan to support nitrogen equilibrium in the adult human subject when fed as supplements in a diet otherwise deficient in tryptophan.

Trials were first made of tryptophan-low basal diets containing a tryptophan-deficient hydrolysate of casein as the chief source of nitrogen, and also containing selected dietary items low in protein as the sources of additional calories and the non-protein requisites. This proved unsatisfactory because tryptophan is so widely distributed, even among dietary items relatively low in nitrogen, as to make it difficult to avoid its incidental inclusion in large enough amounts in this type of diet to prevent the production of a markedly negative nitrogen balance. Synthetic diets were therefore found preferable.

Diets providing 0.1 gm of nitrogen per kilogram per day in the form of an acid-hydrolysate of casein and enough more relatively pure carbohydrates and fats to raise the daily caloric intake to at least 42 Cal. per kilogram were subsequently employed. They contained approximately 25 mg of tryptophan and supported nitrogen equilibrium when supplemented with 200 mg of tryptophan per day. Nitrogen equilibrium was maintained in 15 subjects for periods of 12 days or more with total intakes of L-tryptophan not exceeding 225 mg each per day.

After the preliminary period of 8 to 19 days allowed for adjustment to the supplemented synthetic diet, responses to removal and replacement of the supplement were always fairly prompt, usually immediate, provided that a high caloric intake was maintained.

DL-tryptophan, in the amount of 400 mg per day, promoted responses equivalent to those noted on 200 mg of the L isomer, but the responses to 200 mg of DL-tryptophan per day were suboptimum. Daily supplementation with 200 mg of D-tryptophan failed to maintain nitrogen balance, but in one instance 400 mg per day seemed to promote a borderline response. There is little doubt that in the human subject the capacity of D-tryptophan to provide for maintenance is definitely inferior to that of L-tryptophan, but whether the D isomer could support nitrogen balance adequately, if fed in sufficiently large amounts in diets calorically adequate, is uncertain.

Acetyl-L-tryptophan in daily supplements (240 mg) equivalent to 200 mg of L-tryptophan appeared to be about as well utilized as the latter, but the response to 240 mg of acetyl-D-tryptophan was definitely negative. Acetylation does not enhance the availability of D-tryptophan for maintenance in the human adult.

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# THE MINIMUM RIBOFLAVIN REQUIREMENT OF THE INFANT<sup>1</sup>

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## FOUR FIGURES

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The problem of ascertaining minimum dietary vitamin requirements has been complicated in recent years by the discovery of the antivitamins on the one hand and of the biosynthesis of vitamins by intestinal bacteria on the other. Vitamin requirements are also affected by disease. Hence, in considering requirements it is necessary to define the conditions under discussion. The present study was an attempt to throw light on the minimum riboflavin requirement of the healthy infant on a diet differing little from that customarily given to infants in this country.

## GENERAL PROCEDURE

The general plan adopted in this study was to feed infants a purified diet in which vitamins were supplied almost en-

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tirely in the form of accurately measured supplements. On such a diet the riboflavin content could be accurately controlled and altered at will. It was not deemed justifiable to reduce the riboflavin intake to a point at which symptoms of deficiency appeared. Instead, the attempt was made to determine the minimum riboflavin intake which would maintain the individual in health as judged by laboratory measurements of the riboflavin in blood and urine. Three measurements of blood riboflavin were made: free riboflavin plus flavin mononucleotide of the serum (called hereafter free riboflavin, since only traces of mononucleotide are present), total riboflavin of the white blood cells, and total riboflavin of the red blood cells. The total riboflavin of the serum, which includes the larger flavin adenine dinucleotide fraction, was also measured, but the values are not recorded since no consistent changes were observed throughout the experiment. This suggests that measurement of the total serum riboflavin would be of little help in detecting riboflavin deficiency.

The 24-hour urinary output of riboflavin was measured in order to ascertain the intake which would maintain but not exceed the minimum urinary excretion. The possible significance of this "point of minimum excretion" demands a word of explanation. Some of us (Najjar and Holt), when studying thiamine requirements in adults and adolescents, had found in 1943 that as the intake of this factor was reduced the urinary excretion fell sharply, until a certain critical minimum value was attained beyond which further reductions in intake produced only negligible further decreases in urinary output. This critical "point of minimum excretion" was found to occur at an intake two to three times greater than that which led to symptoms of deficiency. The point of "minimum urinary excretion" of thiamine has been determined for infants as well as older subjects, and has been used in estimating the thiamine requirement of the infant (Holt et al., '49). Further evidence indicating that this critical point corresponds closely with the minimum requirement for the maintenance of health was obtained from studies of tissue thiamine in rats at various levels of intake (Salcedo et al.,



'48). It was found that the brain is incapable of storing surplus thiamine when an excess of thiamine is fed. The thiamine content of the brain remains constant unless the quantity in the diet is reduced below the point of minimum urinary excretion, whereupon it falls sharply.

In the case of riboflavin data are not available correlating a point of critical urinary excretion to clinical manifestations of deficiency. Nevertheless it has been shown in older subjects (Najjar et al., '44) that a reduction in riboflavin intake reduced the urinary riboflavin to a minimum level, and this was not accompanied by clinical evidences of deficiency. The growing rat on a riboflavin deficient diet excretes an exceedingly small amount of riboflavin until sufficient riboflavin to permit maximum growth is provided, at which point the excretion is increased markedly (Burch et al., '48). It is recognized that methods for measuring urinary riboflavin are not entirely specific and that the very small "minimum" excretion on low riboflavin diets may consist in part—perhaps in large part—of non-riboflavin fluorescent substances. If this were the case, however, it would not vitiate the present argument. We therefore undertook to determine the point of minimum riboflavin excretion in the urine of the infant as a guide to the minimum requirement, since this would furnish a readily measurable end point presumably not far above the intake level leading to symptoms of deficiency.

## METHODS

### *Subjects*

Three male infants were used in this study. They were in good health except for mental deficiency. The mental state could be attributed in one to internal hydrocephalus, in the second to neonatal kernicterus; in the third it was without obvious explanation. The ages of these infants were 22, 14 and 32 months and they weighed 5.9, 8.6 and 9 kg, respectively, at the start of the experiment. They were placed on metabolism frames permitting separate collection of urine and feces for 5 days out of every 7 throughout the period of

study. Physical examinations with special reference to signs of riboflavin deficiency were carried out daily. Ophthalmologic examination, which included inspection of both the media and the fundus, and slit lamp examinations were repeated monthly throughout the experiment and sometimes more frequently. The subjects were weighed daily and measurements of length and head and chest circumferences were repeated at monthly intervals. X-rays of the long bones were taken at 4-week intervals and other clinical laboratory data, such as complete blood counts, urinalyses and electrocardiograms, were obtained at frequent intervals.

### *Diet*

This consisted of vitamin-free casein,<sup>5</sup> commercial hydrogenated cottonseed oil,<sup>6</sup> and a vitamin-free dextrimaltose<sup>7</sup> in proportions to yield the following percentage distribution of calories: protein 15%, fat 35%, carbohydrate 50%. Each child received 100 cal. per kilogram of body weight daily. Minerals were supplied in the form of a mineral mixture.<sup>8</sup> Fat soluble vitamins were furnished by Oleum percomorphum,<sup>9</sup> 15 drops daily. The water soluble vitamins<sup>10</sup> were administered as a

<sup>5</sup> Labco. This casein contains 0.35  $\mu\text{g}$  of riboflavin per gram.

<sup>6</sup> Crisco. Contributed by Procter and Gamble.

<sup>7</sup> Specially prepared by Mead Johnson and Company through the courtesy of Dr. Warren M. Cox, Jr.

<sup>8</sup> The mineral mixture, supplied at the level of 5 gm per 750 cal. of diet, consisted of:

	%		%
NaCl	18.9	Fe citrate	2.21
CaHPO <sub>4</sub>	25.0	CuSO <sub>4</sub> (anhydrous)	0.24
MgSO <sub>4</sub> (anhydrous)	6.86	MnSO <sub>4</sub> (anhydrous)	0.15
KHCO <sub>3</sub>	44.4	KI	0.015
KCl	2.88	Na <sub>2</sub> F <sub>2</sub>	0.03

<sup>9</sup> Contributed by Mead Johnson and Company.

<sup>10</sup> The vitamin mixture, calculated in the case of the B factors to supply the same quantities as a quart of fresh cow's milk, gave the following daily quantities:

	$\mu\text{g}$		$\mu\text{g}$
Ascorbic acid	25.0	Inositol	180.0
Thiamine	0.38	Folic acid	.05
Nicotinic acid	0.87	Para-aminobenzoic acid	0.5
Pantothenic acid	3.5	Choline	147.0
Pyridoxine	0.67	Riboflavin	Varied

supplement which was divided into 4 equal portions given at 4 equally spaced intervals during the day.

### *Laboratory measurements*

Riboflavin in the urine was determined by the fluorometric method of Najjar ('41), using a Pfaltz and Bauer fluorophotometer. Total daily urines were collected in amber bottles and preserved with acetic acid. Measurements of the riboflavin in the stools were carried out from time to time to evaluate the factor of biosynthesis by the intestinal bacteria. The stools were preserved by adding 10 ml of glacial acetic acid to each 100 gm of stool. Extractions were performed according to the method of Najjar et al. ('44). Measurements of riboflavin in the red cells, white cells and serum were carried out by a microfluorometric procedure as previously described (Burch, Bessey and Lowry, '48). The bloods samples were drawn from the heel or finger tip.

### *Procedure*

It was felt, in all three of the cases, that time could be saved in approaching the "point of minimum urinary excretion" if riboflavin were completely omitted from the diet for a period instead of being gradually reduced. The complete omission was followed by a reduction in urinary output to a minimum level ranging between 33 and 50  $\mu$ g per day. After these urinary figures had become stable, riboflavin was added to the diet in a stepwise manner until "spilling" in the urine occurred in quantities substantially above these figures. The intake was then alternately reduced and raised in order to determine the quantity ingested which would just sustain urinary excretion above the minimum level.

### RESULTS

The results are presented graphically in the accompanying charts (figs. 1, 2, 3 and 4) and tables. The protocols follow:

*Case I, J. G. (fig. 1)*

This study was a preliminary one which was incomplete and not carried out to its logical conclusion. It gave only an approximation of the range in which the end point lay. Although a significant excretion of riboflavin in the urine did not occur until the intake was increased to 0.6 mg daily, it seems quite likely that this excretion might have taken place if the 0.5 mg intake had been sustained for a longer period of time, since there was some increase in urinary riboflavin on the last day of this period.

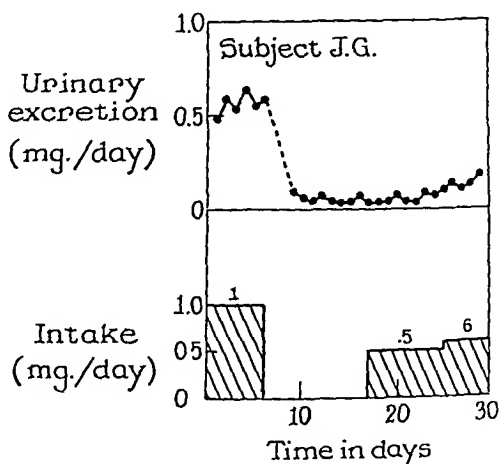


Figure 1

The data obtained thus indicated that the "point of minimum excretion" occurred at an intake of somewhat less than 0.6 mg daily; they were not sufficient to determine a more accurate end point.

At the time of this experiment, the methods for determining riboflavin in blood were not in operation and no data on blood levels were obtained.

*Case II, N. K. (fig. 2)*

After a period of 50 days in which riboflavin was entirely omitted and urinary excretion varied between 35 and 50  $\mu$ g,

it was introduced again in increasing amounts until higher urinary figures were obtained. A gradual titration of the point of minimum urinary excretion was then carried out by adjusting the riboflavin intake until the minimum urinary excretion values were just exceeded. In this subject 0.40 mg of riboflavin daily gave the desired effect.

Blood determinations were not made at the onset of this study, the first being made on the 50th day of riboflavin withdrawal. Because of the delay in subsequently building up the patient to the point of "spilling," it is reasonable to conclude that the riboflavin-free period depleted the subject appreciably beyond the point of minimum excretion. It is noteworthy that at this time low values of free riboflavin in the serum and of total riboflavin in both the red and white cells were observed.

It is perhaps not possible to state with confidence the normal range for riboflavin in the elements of the blood. Burch, Bessey and Lowry ('48) reported for a dozen "well nourished" adults the following values: white cells, 252  $\mu\text{g}\%$  (range 227-293), red cells, 22  $\mu\text{g}\%$  (range 18-26), free riboflavin of the serum, 0.8  $\mu\text{g}\%$  (range 0.3-1.3). Data from infants receiving a normal infant diet showed values in the same range. The infant data are used as the basis for the "normal range" of figures 2 and 3. During the period of depletion the free riboflavin of the serum of infant N. K. was less than 0.1  $\mu\text{g}\%$ , and remained so for two weeks in spite of an intake of 0.4 to 0.8 mg riboflavin per day and in spite of a little "spillage" of riboflavin in the urine. However, with an average intake of about 0.35 mg per day the serum values gradually rose to 0.5 mg% after two months of realimentation. Similarly, the white cell and red cell values rose slowly. The white cell values appeared normal after two months, and the red cells after three. Thus, regardless of the significance of the concept of minimum point of excretion, this infant was apparently restored and maintained in riboflavin equilibrium with 0.4 mg riboflavin per day.

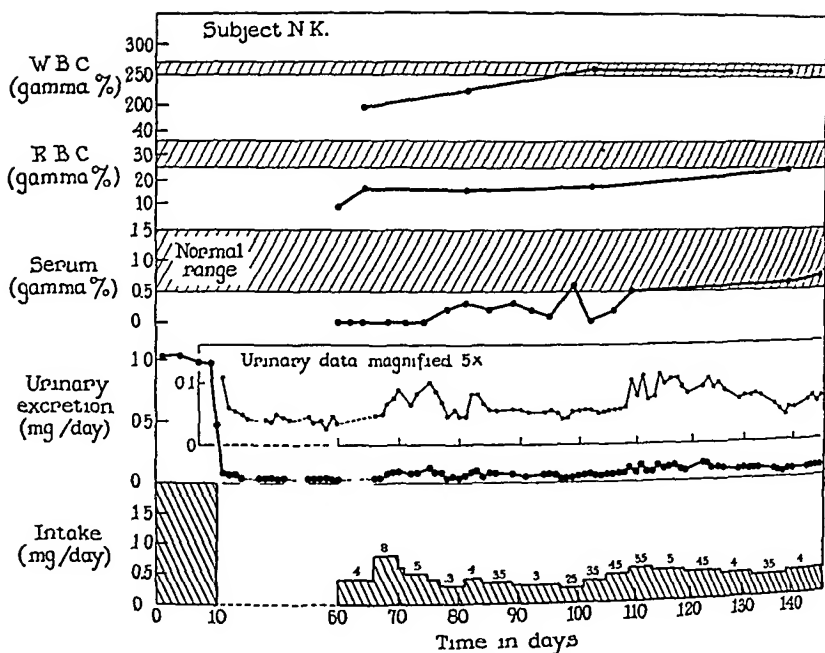


Figure 2

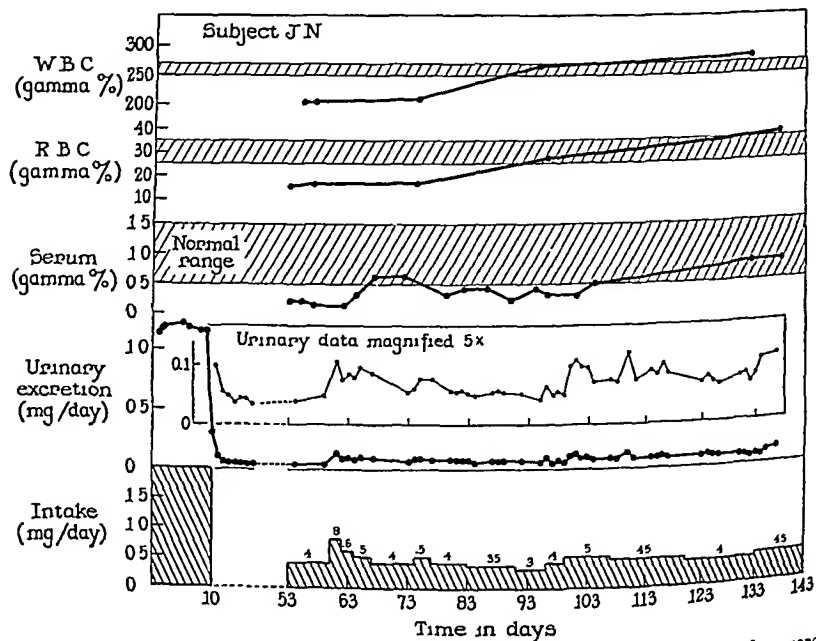


Figure 3

The term "normal" here refers to figures obtained with infants who were receiving a normal or conventional infant diet.

*Case III, J. N. (fig. 3)*

Riboflavin was withdrawn for 43 days and this resulted in urinary excretions varying between 33 and 40  $\mu\text{g}$  daily. As previously described, "spilling" was obtained by increasing the riboflavin dosage and then carefully increasing and decreasing the amount ingested until the urinary output just exceeded the minimum figures. This point corresponded to an intake of 0.4 mg riboflavin daily in this subject also.

Blood studies were begun in this subject on the 43rd day without riboflavin. The initial values at the end of this period of depletion were comparable to those for the previous infant. The serum values for free riboflavin were a trifle higher but remained in general below 0.5  $\mu\text{g}\%$  until the white cells and red cells were restored to "normal," an interval of a month or two.

Figure 4 gives a composite picture of the average urinary riboflavin values occurring at the different levels of intake for all three subjects. Although the slope of the curve based on riboflavin excretion plotted against intake continues to increase up to an intake of 0.8 mg daily, it is clear that the beginning of the increased slope occurs at an intake of approximately 0.4 mg per day. We are inclined to interpret the higher excretion which begins at this level as evidence of a surplus of riboflavin beyond the requirements of the body.

*Riboflavin load tests.* These tests were performed at intervals on one of the above patients (subject J. G., weight 10 kg) using the procedure of Najjar and Holt ('41)—measuring the 4-hour excretion in the urine after the intravenous administration of 16  $\mu\text{g}$  of riboflavin per kilogram. They illustrate a progressive retention of riboflavin after the "point of minimum excretion" is attained, and indicate that the load test has the advantage of revealing various degrees of deficiency beyond that point. Retentions in these studies varied from 99% to 68% of the 160  $\mu\text{g}$  test dose. The greater retention occurred after depletion and was accompanied by a two-hour control excretion of 2.7  $\mu\text{g}$  and a total 4-hour test

excretion of 7.8  $\mu\text{g}$ . The smaller retention followed adequate intake of riboflavin; at this time the control value was 6  $\mu\text{g}$  and the 4-hour figure was 63  $\mu\text{g}$ .

Because of the possible contribution of intestinal bacteria to the riboflavin balance, the *riboflavin content of the stools* was studied routinely in our first subject and some isolated

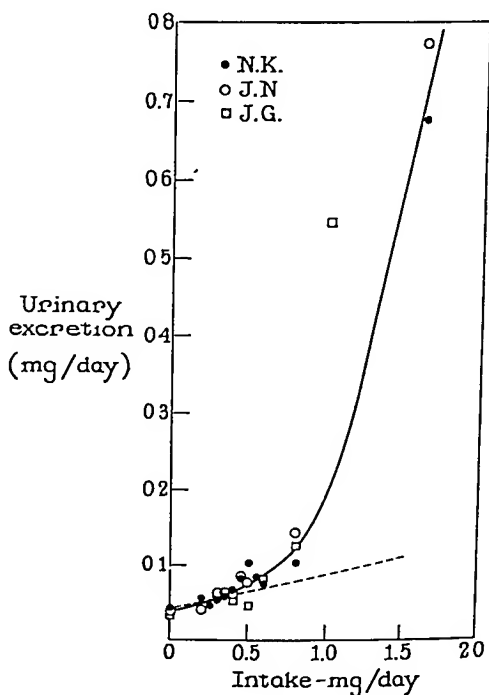


Figure 4

measurements were made in the other two infants. These observations are recorded in table 1. The total daily riboflavin content of the stool was found to vary widely in proportion with the stool weight, but the riboflavin concentration per gram of fresh stool remained singularly constant regardless of the reduction in intake to zero. It is thus obvious that biosynthesis of riboflavin occurs in the intestine of the infant, as in that of the adult. How much of this riboflavin is avail-



able for absorption cannot be ascertained from present data. To determine this point requires an effective method of inhibiting the growth of the organisms which synthesize riboflavin. The total stool excretion, however, is only a 6th of the amount that the present data suggest is necessary for maintenance.

The infants in these experiments made satisfactory gains in weight except toward the end of the study, when a flattening out of the weight curve occurred in each instance. The

TABLE 1  
*Riboflavin content of the stool in relation to intake*

SUBJECT	INTAKE	DAILY AMOUNT, AVER.	CONTENT OF STOOL		
			Aver. at each intake level	Concentration	
				Average	Aver. at each intake level
	<i>mg/day</i>	<i>μg</i>	<i>μg</i>	<i>μg</i>	<i>μg</i>
J.G.	1.0	45.7 <sup>1</sup>		2 <sup>1</sup>	
		84.7		4.5	
		98.9		2.3	
		62.4	75.3	5.1	3.5
	0.6	40.3		5.4	
	0.5	80		4.2	
	0.4	86.5		6.0	
		76		5.0	
				4.0	
		73.4	78.6	4.8	5.0
	0.3	66.3		4.5	
		68.9	67.2	4.7	4.6
	0.0	67.2		5.0	
		60.5		5.0	
		63.9		4.2	
		145.1	84.2	5.6	5.0
N.K.	0.0	120		6.9	
		34.7	77.3	3.9	5.4
J.N.	0.0	22.8		2.5	

<sup>1</sup> Each of these figures represents the average of 5 individual daily figures.

thought that this failure to gain weight might be due to some degree of riboflavin deficiency, even though our chemical criteria in the blood and urine were satisfied, naturally occurred to us. Other evidence, however, has led us to dismiss this view and to attribute the phenomenon to some unknown deficiency in our purified diet. In the first place, we have noticed that all infants placed on our purified diet will thrive for only about 6 months, regardless of the vitamin mixture supplied; failure to gain weight will occur even on high riboflavin intakes, and in our subsequent experiments we now make a point of completing our observations early before this unknown deficiency manifests itself. Our observations on this unknown type of deficiency, which has been found to respond to brewers' yeast, are presented elsewhere. A second reason for exonerating riboflavin deficiency of responsibility for the development of stationary weight in the experiments reported here is the fact that in subsequent experiments carried out for another purpose we have been able to attain riboflavin equilibrium at the point of minimum excretion in a shorter period of time, and have been able to duplicate the urinary and blood findings here reported without encountering the arrest of weight.

#### DISCUSSION

The data reported above indicate that in infants weighing 5.9 to 9 kg, fed as described, an intake of riboflavin of 0.4 to 0.5 mg per day is compatible with freedom from symptoms of riboflavin deficiency, maintenance of urinary riboflavin excretion at the critical point (the "point of minimum excretion"), and maintenance of presumed normal levels of riboflavin in the blood serum, red cells, and white cells. They do not permit one to state what the absolute minimum requirement is, even under these defined conditions.

The data suggest that the measurement of red cell riboflavin may be of service in assessing the riboflavin nutritional status. These values during depletion were lowered to less than half the probable normal value. The percentage change

was greater than for the white cells, which are also more difficult to measure. The serum values for free riboflavin might be useful, except that in the deficient zone the absolute values are exceedingly low and therefore difficult to measure with any accuracy.

We wish to emphasize the fact that we are not undertaking to recommend specific dietary allowances of riboflavin for infants on the basis of the data at hand. As we pointed out at the start, a requirement established by laboratory methods applies only to limited and well-defined conditions. On different diets and in various pathological states requirements may be quite different. Furthermore, any recommended allowance involves the further task of establishing a margin of safety, the proper size of which is an open question.

#### SUMMARY

An intake of 0.4 mg of riboflavin per day by the infant served to maintain adequate blood levels, to maintain the urinary excretion at the upper limit of the zone of minimum excretion, and was not associated with clinical evidences of deficiency.

The limitations of these studies and the relation of the above figure to the minimum riboflavin requirements of the infant are discussed.

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# VITAMIN A UTILIZATION STUDIES

## I. THE UTILIZATION OF VITAMIN A ALCOHOL, VITAMIN A ACETATE AND VITAMIN A NATURAL ESTERS BY THE CHICK

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The present authors have undertaken a series of studies on vitamin A utilization with the particular purpose of elaborating the manner in which certain factors may affect differently the biological utilization of various esters and the free form of vitamin A.

Several investigators (Emmett and Bird, '37; Gray, Hickman and Brown, '40; Sobel et al., '48) have reported comparisons between the utilization of vitamin A esters and vitamin A alcohol under various experimental conditions. Their results indicate that the amount of the vitamin absorbed through the intestinal wall, and thereby made available to serve its essential physiological function in the metabolic processes of the animal, is dependent on a number of factors in addition to the quantity of vitamin A fed. These factors may be considered to fall into two groups, namely: (1) factors affecting the stability of the vitamin *in vivo* prior to absorption through the intestinal wall; and (2) agents or conditions which influence the intestinal absorption per se.

With respect to the factors affecting the stability of the vitamin, the interrelationship of vitamins A and E has been emphasized by Hickman et al. ('44), Gridgeman ('44) and Lemley et al. ('47). Their investigations are pertinent to the present study, however, only insofar as vitamin E may influence the utilization of vitamin A prior to its passage as the

free, unesterified form through the intestinal wall into the lymphatics and blood stream.

Studies on the mechanisms and mode of vitamin A absorption have been reported by Gray, Morgareidge and Cawley ('40), Clausen ('43) and Popper and Volk ('44). These investigations have shown that normally vitamin A esters are hydrolyzed prior to absorption but that, under certain physiological conditions which adversely affect the supply of the pancreatic enzymes to the duodenal contents, these esters are not hydrolyzed and absorption suffers.

It is therefore apparent that there exists a third group of factors, exerting favorable or adverse effects on the physiological mechanism of vitamin A ester hydrolysis, which would affect biological comparisons of dissimilar chemical forms of vitamin A.

We have emphasized in the present study the investigation of factors affecting the hydrolysis of vitamin A esters, particularly in respect to their varying effects on the utilization of differing forms of vitamin A as determined by liver storage measurements.

Chicks were used as the experimental species in the first part of our studies because of their relatively poor ability to utilize fats. We believed that for this reason factors affecting vitamin A ester hydrolysis could be more easily demonstrated with chicks than with other available experimental animals.

## METHODS

### *Preparation of vitamin A concentrate*

Vitamin A alcohol, acetate and natural ester concentrates were prepared in the laboratory, using a male soupfin shark liver oil as the source of vitamin A.

The vitamin A alcohol concentrate was obtained by saponifying the soupfin liver oil with alcoholic potassium hydroxide, extracting the unsaponifiable fraction with peroxide-free ethyl ether, and removing the solvent under reduced pressure. The vitamin A acetate was prepared by acetylating a portion of

the vitamin A alcohol. The natural ester concentrate was made by a process developed in our laboratories which does not involve the use of high vacuum distillation.

As the experiments described in this paper were conducted over an extended period of time, fresh preparations of the respective concentrates were made at various intervals during the course of the investigation. Since standardized procedures were used for their preparation, the various concentrates were extremely uniform in regard to spectrophotometric and chemical characteristics (table 1).

TABLE 1

*Physico-chemical characteristics of vitamin A concentrates fed*

PHYSICO-CHEMICAL CHARACTERISTICS	SOUFFIN SHARK-LIVER OIL	VITAMIN A ALCOHOL	VITAMIN A ACETATE	VITAMIN A NATURAL ESTER
$E_{1\text{ cm}}^{1\%}$ 328 m $\mu$	66.0	890-915	740-753	304-335
Ratio = $\frac{E_{300\text{ m}\mu}}{E_{328\text{ m}\mu}}$	0.67	0.64-0.65	0.66-0.68	0.66-0.69
Irrelevant absorption (%)	10.0	6.0-8.0	6.0-9.0	8.0-11.0
$L_{1\text{ cm}}^{1\%}$ 620 m $\mu$	155.0	2140-2200	1600-1630	725-800
Vitamin A alcohol (%)		99.0-100	2.0-4.0	3.0-6.0
Vitamin A acetate (%)			96.0-98.0	
Vitamin A natural ester (%)	100.0	0.0-1.0		94.0-97.0

The ultraviolet spectrophotometric measurements were made on a Beckman spectrophotometer, using absolute ethanol as a solvent. The proportion of irrelevant absorption present in the spectrophotometric curves in the region of the vitamin A absorption maximum was calculated according to the method devised by Morton and Stubbs ('46), using the fixation wave lengths specified for the solvent ethanol. The  $L_{1\text{ cm}}^{1\%}$  620 m $\mu$  values were measured on an Evelyn photoelectric colorimeter according to the procedure of Dann and Evelyn ('38). Percentages of the alcohol and ester forms of vitamin A in the respective concentrates were determined by the procedures

described by Reed et al. ('44). The concentrates were stored under nitrogen and refrigeration.

### *Preparation of vitamin A dilutions for feeding*

Vegetable oils and other materials used as diluents were high quality commercial and pharmaceutical products. The vegetable oils contained a minimum of peroxides and showed no evidences of rancidity.

The dilutions of the vitamin A concentrates for feeding were calculated on the basis of spectrophotometric units of vitamin A ( $E_{1\text{ cm}}^{1\%} 328 \text{ m}\mu \times 2,000$ ). The dilutions were assayed before and after the test feeding period to determine if any change in potency had taken place.

### *Basal diet*

White Leghorn and New Hampshire chicks were used according to their availability for the experiments. The one-day old chicks were placed on a low vitamin A diet (Almquist et al., '43), with several modifications. The composition of the basal ration is shown in table 2.

The incorporation of the indicated amount of vitamin A into the diet was sufficient to maintain the chicks at a level at which no visible symptoms of vitamin A deficiency were apparent, and no significant storage of the vitamin A occurred in the liver.

The chicks were housed in electrically heated battery brooders under uniform conditions of light, temperature, and so forth. The basal feed and water were supplied ad libitum. At 50 to 56 days of age the chicks were divided into comparable groups according to sex and weight and placed on test.

### *Vitamin A liver storage measurements*

The livers from each group of chicks were weighed individually, pooled and homogenized in a Waring Blendor, and samples were taken for the determination of vitamin A. A



modified procedure based on the method described by Glover et al. ('47) was used for all liver assays.

A 5- to 10-gm sample of liver tissue was thoroughly mixed with approximately 4 times its weight of anhydrous sodium sulfate. The mixture was extracted with 4 portions, of 50 ml each, of redistilled petroleum ether (B.P. 40 to 60°C.). The extract was filtered into a 250-ml volumetric flask, the filter paper being washed with small portions of petroleum ether

TABLE 2  
*Composition of the basal ration*

BASAL MIXTURE		VITAMIN SUPPLEMENTS	
	%		<i>per lb. basal mixture</i>
Fish meal (extracted sardine meal)	15.00	Vitamin D (Delsterol)	454 A.O.A.C. units
Brewers' yeast	7.50	Vitamin K (2-methyl naphthoquinone)	0.18 mg
Wheat bran	10.00	Vitamin E (21.3% mixed tocopherols)	11.0 mg
Oyster shell	0.50	Vitamin A (shark liver oil)	1,200 spec. units
Salt	1.00		
Cottonseed oil (Wesson)	2.00		
Ox bile salts	0.20		
Rice flour (polished rice)	63.54		
Choline chloride	0.20		
Manganese sulfate (85%)	0.06		
	100.00		

to remove traces of vitamin A. The volume was adjusted to 250 ml and a suitable aliquot taken and evaporated to dryness by a stream of nitrogen. The residue was dissolved immediately in 1 ml of chloroform and the vitamin A estimated by the Carr-Price reaction, using an Evelyn colorimeter equipped with a 620 mμ filter. The colorimeter was calibrated against the U.S.P. Vitamin A Reference Standard for determining the vitamin A values reported. This calibration was found to be the same as when the instrument was calibrated using the

soupfin shark liver oil described above at an assumed potency of  $E_{1\text{ cm}}^{1\%} 328 \text{ m}\mu \times 2,000$ .

We found that the livers of the chicks contained insignificant amounts of the carotinoid pigments, and therefore no corrections for the presence of these compounds were necessary in computing the vitamin A content of the livers.

The vitamin A values reported in this investigation are the result of duplicate determinations which agreed within the limits of  $\pm 3\%$ .

The liver samples were stored under refrigeration until assayed for vitamin A. Control analyses made of various samples indicated no significant destruction of vitamin A during the period of storage.

### *Experiment 1*

This experiment was originally part of a series designed to observe the effects of certain anti-oxidants on the relative utilization of the alcohol, acetate and natural ester forms of vitamin A. In addition, the effect was studied of administering various quantities of an oxidized diluent oil together with the respective vitamin A preparations. A full discussion of these specific considerations is beyond the scope of the present paper and will be presented elsewhere. However, since the data derived from this particular experiment provide an indication of the inhibiting action of relatively large amounts of diluent fat on the utilization of vitamin A esters, the experiment is included in this report.

White Leghorn chicks, 50 days of age, were assigned to experimental groups of 9 each. The dilutions of the respective vitamin A concentrates in cottonseed oil and oxidized corn oil were administered on three successive days, 0.1 ml or 2.0 ml of the respective supplement being fed each day orally by means of a calibrated tuberculin syringe. Each chick received a total of 27,600 units of vitamin A for the three-day period. On the 4th day, or 72 hours after receiving the first supplement, the chicks were killed and the livers removed. The livers of each

group were combined and the vitamin A content determined on the composite sample. The vitamin A liver storage measurements are listed in table 3.

The oxidized corn oil used as a diluent was prepared by aerating the oil for 24 hours at room temperature. Fresh dilutions of the vitamin A preparations were made daily in order to prevent destruction of vitamin A by oxidation.

### Experiment 2

New Hampshire chicks, 52 days of age, were divided into comparable groups of 10 chicks each. Dilutions of the alcohol,

TABLE 3  
Experiment 1 — Vitamin A liver storage by the chick<sup>1</sup>

DILUENT	COTTONSEED OIL NO ANTI-OXIDANTS			COTTONSEED OIL WITH ANTI-OXIDANTS <sup>2</sup>			AERATED CORN OIL NO ANTI-OXIDANTS			AERATED CORN OIL NO ANTI-OXIDANTS		
Volume and potency of suppl. fed daily	0.1 ml @ 92,000 units/ml			0.1 ml @ 92,000 units/ml			0.1 ml @ 92,000 units/ml			2.0 ml @ 4,600 units/ml		
Total units fed in 3-day period	27,600			27,600			27,600			27,600		
Group no.	1	2	3	4	5	6	7	8	9	10	11	12
Form of vitamin A	Alco- hol	Acet- ate	Ester	Alco- hol	Acet- ate	Ester	Alco- hol	Acet- ate	Ester	Alco- hol	Acet- ate	Ester
Average wt. of livers (gm)	16.9	16.4	17.5	15.8	15.1	18.6	16.5	16.8	15.7	16.2	18.3	14.6
Units of vitamin A per liver	6,352	9,422	8,680	6,178	9,362	8,128	6,600	7,963	7,018	7,776	7,851	6,220
Vitamin A stored (%)	23.0	34.1	31.4	22.4	33.9	29.4	23.9	28.9	25.4	28.2	28.4	22.5

<sup>1</sup> Control groups, receiving diluent oils only as a supplement, showed no liver storage of vitamin A.

<sup>2</sup> Nordihydroguaiaretic acid 0.01% and citric acid 0.05%.

TABLE 4

*Experiment 2 — Liver storage in chicks fed daily supplement of 10,000 units vitamin A in various diluent oils<sup>1</sup>*

DILUENT	COTTONSEED OIL	CORN OIL	SARDINE OIL	BASKING SHARK OIL	MINERAL OIL U.S.P.										
Vol. and potency of suppl. fed daily	2.0 ml @ 5,000 units/ml	2.0 ml @ 5,000 units/ml	2.0 ml @ 5,000 units/ml	2.0 ml @ 5,000 units/ml	2.0 ml @ 5,000 units/ml										
Total units fed in 3-day period	30,000	30,000	30,000	30,000	30,000										
Group no.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Form of vitamin A	Alco- hol	Acet- ate	Acet- ate	Alco- hol	Alco- hol	Acet- ate	Alco- hol	Acet- ate	Ester	Alco- hol	Acet- ate	Ester	Alco- hol	Acet- ate	Ester
Average wt. of livers (gm)	17.8	18.0	17.4	17.8	16.8	18.6	17.4	18.3	16.7	18.8	18.0	18.6	19.6	17.9	17.0
Units of vitamin A per liver	7,490	6,830	5,320	7,150	5,970	4,940	3,930	3,960	1,390	4,510	2,390	1,880	4,860	2,160	1,465
Vitamin A stored (%)	25.0	22.8	17.7	23.8	19.9	16.5	13.1	13.2	4.6	15.0	8.0	6.3	16.2	7.2	4.9

<sup>1</sup> Control groups, receiving diluent oils only as a supplement, showed no liver storage of vitamin A.

acetate and natural ester concentrates containing 5,000 vitamin A units per milliliter were made in cottonseed oil, corn oil, sardine oil, basking shark oil and mineral oil. The supplements contained 0.01% by weight of hydroquinone as an anti-oxidant.

Each chick received orally 2.0 ml of a vitamin A supplement daily for three successive days, or a total of 30,000 units of the vitamin. The chicks were sacrificed on the 4th day and the livers composited and assayed for vitamin A as described for the preceding experiment. The results are shown in table 4.

### *Experiment 3*

White Leghorn cockerels, 56 days of age, were assigned to groups of 12 chicks each. Dilutions of the respective vitamin A forms containing the specified units of vitamin A per milliliter were prepared in corn oil, castor oil, jojoba seed oil and ethyl laurate. The composition and chemical characteristics of jojoba seed oil are described by Jamieson ('43). The supplements contained 0.5% by weight of mixed tocopherols as an anti-oxidant.

The chicks received their daily supplements of the vitamin A-containing preparations as described for the previous experiments. A total of 30,000 vitamin A units was administered to each chick for the three-day test period.

The livers from each group of chicks were divided into 4 subgroups containing three livers each and assayed for vitamin A. The results are listed in table 5.

The data derived from this experiment were analyzed statistically in order to determine the mathematical significance of the values obtained.

### DISCUSSION OF RESULTS

The experimental results, which are discussed in detail in the following paragraphs, clearly demonstrate the existence of factors affecting the hydrolysis of the ester form of vitamin A at high levels of intake under various test conditions. The

TABLE 5

Experiment 3 — Relationship between chemical forms of vitamin A and diluent oils, as measured by liver storage by the chick<sup>1</sup>

DILUENT	CORN OIL			CORN OIL			CASTOR OIL		
	1	2	3	4	5	6	7	8	9
Volume and potency of suppl. fed daily	2.0 ml @ 5000 units/ml			0.1 ml @ 100,000 units/ml			2.0 ml @ 5000 units/ml		
Total units fed in 3-day period	30,000			30,000			30,000		
Group no.									
Form of vitamin A fed	Alcohol	Acetate	Ester	Alcohol	Acetate	Ester	Alcohol	Acetate	Ester
Sub-group i (units)	10,105	9,718	6,507	10,293	10,604	8,275	7,269	7,444	4,560
Sub-group ii (units)	12,657	10,743	8,720	11,390	11,751	7,878	7,592	8,852	4,117
Sub-group iii (units)	11,474	12,366	7,162	11,224	11,598	8,024	9,002	8,212	5,684
Sub-group iv (units)	8,159	7,482	6,134	8,614	12,804	9,109	8,972	7,582	5,994
Avg. vit. A per liver	10,667	10,077	7,131	10,380	11,689	8,322	8,209	8,023	5,089
Avg. vit. A stored (%)	35.6	33.6	23.8	34.6	39.0	27.7	27.4	26.7	17.0
DILUENT	JOJOBA SEED OIL			JOJOBA SEED OIL			ETHYL LAURATE		
	10	11	12	13	14	15	16	17	18
Volume and potency of suppl. fed daily	2.0 ml @ 5000 units/ml			0.1 ml @ 100,000 units/ml			2.0 ml @ 5000 units/ml		
Total units fed in 3-day period	30,000			30,000			30,000		
Group no.									
Form of vitamin A fed	Alcohol	Acetate	Ester	Alcohol	Acetate	Ester	Alcohol	Acetate	Ester
Sub-group i (units)	8,540	4,584	3,133	9,379	10,321	7,112	6,969	4,462	3,400
Sub-group ii (units)	9,694	4,354	2,028	8,834	10,606	7,708	9,550	4,518	4,266
Sub-group iii (units)	10,291	4,972	2,341	8,400	11,043	10,205	9,143	4,807	4,217
Sub-group iv (units)	10,876	5,157	2,555	10,052	9,990	8,565	9,648	3,170	2,744
Avg. vit. A per liver	9,850	4,767	2,439	9,166	10,490	8,398	8,828	4,239	3,657
Avg. vit. A stored (%)	32.8	15.9	8.1	30.5	35.0	28.0	29.4	14.1	12.2

<sup>1</sup> A difference of 1,507 is required for significance (5% level) between any two of the following test groups: 1, 2, 3, 7, 8, 9, 10, 11, 12, 16, 17 and 18. A difference of 1,943 is required for significance (5% level) between any two of the following test groups: 1, 2, 3, 4, 5, 6, 10, 11, 12, 13, 14 and 15.

Control groups, receiving diluent oils only as a supplement, showed no liver storage of vitamin A.

importance of these factors at lower levels of vitamin A intake was also demonstrated in preliminary and additional experiments performed in this laboratory. The earlier tests were designed primarily to determine the vitamin A dosage required for optimum liver storage of the vitamin. In feeding 2,500 units daily, as compared to the 10,000 unit level fed in the experiments described in this paper, the differences in availability of the various forms of vitamin A due to hydrolytic factors were of the same order. However, further investigations are necessary to establish definitely the quantitative effects of these factors at the vitamin A levels of intake recommended for normal nutrition.

### *Experiment 1*

Although the significance of the results shown in table 3 cannot be demonstrated by statistical treatment, the data are remarkably uniform and show the same relative utilization of the three forms of vitamin A in all the groups fed a 0.1 ml supplement. The data indicate that factors which might be expected to influence stability *in vivo*, i.e., anti-oxidants and pro-oxidants, actually had little or no effect. The slightly lesser utilization of the natural ester and acetate forms in the aerated corn oil as compared to the cottonseed oil dilutions seems to be due to normal characteristics of the corn oil (see also experiment 2) rather than to the presence of peroxides. This observation with respect to the relatively small importance of factors affecting stability *in vivo* in vitamin A utilization under the conditions of these experiments is also confirmed by a number of other exploratory experiments performed in this laboratory.

The second point of interest is the reversal of the relative utilization of the alcohol and natural ester forms as between the 0.1 ml and the 2.0 ml dilutions in corn oil.

### *Experiment 2*

Foy and Morgareidge ('47), in their investigations concerning the Guggenheim and Koch ('44) method of bioassay for

vitamin A, have demonstrated that variations in vitamin A storage in the livers of rats can be produced by varying the diluent in which the vitamin A dosage is fed. Our experiments confirm that this observation is also true for chicks.

A statistical analysis of the data in table 4 shows that the direct effects of the form of vitamin A, and of the vegetable oils versus the other carriers, exceeded the interaction between these two factors very significantly.

It is to be noted that the utilization of each of the three vitamin forms is only slightly affected by the use of corn oil as a diluent instead of cottonseed oil.

TABLE 6  
*Experiment 2 — Analysis of variance of vitamin A liver storage  
data listed in table 4*

SOURCE OF VARIATIONS	DEGREES OF FREEDOM	MEAN SQUARE	VARIANCE RATIO
Form of vitamin A	2	8,380,305	23.64
Vegetable oils vs. others	1	39,983,335	112.79
Other comparisons among carriers	3	184,579	0.52
Form of vitamin A $\times$ carrier	8	354,501	1.00

On the other hand, the use of sardine body oil, basking shark liver oil or mineral oil has a marked effect on the utilization of all three forms with respect to their utilization from cottonseed oil diluent. The adverse effect of these carriers is more marked in the cases of the vitamin A acetate and vitamin A natural ester than it is in the case of the vitamin A alcohol, except for vitamin A acetate in sardine oil.

Sardine body oil, basking shark oil and mineral oil were chosen because we believed they might have an adverse effect on the hydrolysis of vitamin A esters. It is apparent that they do, although they also inhibit absorption per se. We believe that the mechanism of interference with vitamin A ester hydrolysis is different in the case of sardine oil than in those of basking shark liver oil and mineral oil, being connected in the first case with the presence of glycerides of unsaturated



fatty acids and in the second with the presence of large quantities of hydrocarbons.

### *Experiment 3*

The data in table 5 were analyzed statistically in two separate treatments. An analysis of variance was made of the results for groups 1 through 3, 7 through 12, and 16 through 18. A separate analysis of variance was made of the results for groups 1 through 6 and 10 through 15. These treatments are illustrated in table 7.

TABLE 7

*Experiment 3 — Analyses of variance of vitamin A liver storage data shown in table 5*

SOURCE OF VARIATIONS	DEGREES OF FREEDOM	MEAN SQUARE	VARIANCE RATIO
(1) Groups 1, 2, 3, 7, 8, 9, 10, 11, 12, 16, 17 and 18			
Carrier (diluent)	3	35,976,381	56.67
Form of vitamin A	2	92,754,103	146.10
Sub-groups	3	3,557,119	5.60
Carrier $\times$ form of vitamin A	6	7,797,712	12.28
Pooled error	33	1,097,958	1.00
(2) Groups 1, 2, 3, 4, 5, 6, 10, 11, 12, 13, 14 and 15			
Carrier (diluent)	1	57,693,445	31.21
Form of vitamin A	2	52,368,750	28.33
Volume of supplement	1	60,871,561	32.93
Carrier $\times$ volume	1	23,978,614	12.97
Form of vitamin A $\times$ volume	2	22,492,444	12.17
Pooled error	40	1,848,383	1.00

Comparisons may be made of the utilization in the various carriers of all forms of vitamin A taken together. Comparisons may also be made of the relative utilization of the three forms of vitamin A, taking all types of carrier together. Such comparisons are, however, of minor practical value. Comparison between various pairs of groups is the most valuable for the purpose of drawing inferences.

In the case of groups 1 through 3, 7 through 12, and 16 through 18, it is possible to make 66 comparisons of two groups taken at a time. Forty-nine of these disclose statistically significant differences; 17 do not. Of the 49, 20 comparisons are believed by us to be useful, namely: group 1 with 3, 7 and 16; 2 with 3, 8, 11 and 17; 3 with 9, 12 and 18; 7 with 9 and 10; 8 with 11; 9 with 12; 10 with 11 and 12; 11 with 12; and 16 with 17 and 18. Of the 17 comparisons which do not disclose a statistically significant difference, we believe the following to be useful: 1 with 2 and 10, 7 with 8 and 16, 9 with 18, 10 with 16, 11 with 17, 12 with 18 and 17 with 18.

A typical and valuable line of reasoning which can be applied to these comparisons follows:

1. In the comparisons of group 1 with 3, 7 with 9, 10 with 12 and 16 with 18, the utilization of vitamin A alcohol is compared to the utilization of vitamin A natural esters in the identical carrier. As vitamin A esters are hydrolyzed prior to absorption, the effect of a particular carrier on absorption per se would be the same with respect to vitamin A alcohol as with respect to vitamin A esters. As the esters have been shown by other investigators to be more stable *in vivo* than vitamin A alcohol, their significantly lower utilization in each of the mentioned comparisons can be due only to factors having to do with hydrolysis.

2. The comparison of group 1 with group 10 shows that jojoba seed oil does not have a significant depressive effect on the utilization of vitamin A alcohol as compared with corn oil. As hydrolysis is not involved here, it is shown that the difference between the combined effect of the stability and absorption per se factors in jojoba oil versus that of the same factors in corn oil is not significant.

3. In the comparisons of group 2 with group 11 and group 3 with group 12, absorption per se factors would have the same effect as in the comparison of groups 1 and 10, while stability factors would have even less effect. Therefore, the very significant lowering in the utilization of acetate and natural ester forms in the jojoba oil versus the corn oil dilutions is

shown to be due to the presence of more anti-vitamin A hydrolysis factors (qualitatively or quantitatively) in the jojoba oil.

Following similar lines of reasoning, it can be shown that the inferior utilization of all forms of vitamin A from ethyl laurate versus corn oil demonstrates: (a) greater inhibition of hydrolysis by the ethyl laurate than by corn oil; and (b) the former's adverse effect on stability *in vivo* or absorption per se or both.

Castor oil under the conditions of these experiments is shown to be inferior to corn oil as a diluent in vitamin A feeding, due to factors affecting stability *in vivo* or absorption per se, or both, with the latter factor almost certainly the more important.

In case of groups 1 through 6, and 10 through 15, it is possible to make 66 comparisons of two groups taken at a time. Thirty-seven of these comparisons disclose statistically significant differences. Of these 37, the most important (eliminating duplicate comparisons from the first group-set) are groups 4 with 6, 5 with 6, 12 with 15 and 14 with 15. Of the 29 comparisons which do not disclose statistically significant differences, the following are also of special interest: group 1 with 4, 2 with 5, 3 with 6, 4 with 5 and 13, 5 with 14, 10 with 13, and 13 with 14 and 15.

It happens that every comparison duplicated in the two group-sets, if significantly different according to the first criterion (1,507 units), is also statistically significant according to the second criterion (1,943 units).

A reduction in the volume of the jojoba seed oil from 2.0 ml to 0.1 ml significantly increases the utilization of vitamin A acetate and vitamin A natural ester. As the same change in volume of jojoba seed oil has no significant effect on the utilization of vitamin A alcohol, it can be seen that the improved utilization of the ester forms in the 0.1 ml dilutions is due to the lesser amounts of hydrolysis-inhibiting factors carried in this quantity of diluent.

Although the differences between the 2.0 ml and 0.1 ml corn oil dilutions with respect to utilization of the various forms of the vitamins are not statistically significant, there is indication that the reduction of corn oil volume similarly tends to reduce the already small amount of hydrolysis-inhibiting factors present.

The comparisons of group 1 with group 4 and groups 10 with 13 show that factors affecting absorption per se and stability are of minor importance in both corn oil and jojoba oil, unless these factors operate in opposing directions with substantially equal magnitude.

#### SUMMARY

1. Vitamin A liver storage tests were used as the criterion for determining the relative utilization of the alcohol, acetate and natural ester forms of vitamin A by the chick under various experimental conditions.

2. Wide variations in the storage of vitamin A in the livers of chicks were found, depending both on the form of vitamin A fed and on the character and quantity of diluent. The experimental data were subjected to analyses of variance, and the statistical significance of a great part of the data was established. The importance was demonstrated of factors affecting the hydrolysis of vitamin A esters in vitamin A utilization. The following carriers were shown to contain factors inhibiting vitamin A ester hydrolysis: jojoba seed oil, ethyl laurate, basking shark liver oil and mineral oil. Sardine oil was shown to contain factors which interfere with the hydrolysis of vitamin A natural esters, although not with the hydrolysis of vitamin A acetate. Although statistical significance was not demonstrated, it appears likely that cottonseed oil and corn oil also contain small amounts of factors which interfere with vitamin A ester hydrolysis.

The existence of factors affecting vitamin A absorption per se was also demonstrated.

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# VITAMIN A UTILIZATION STUDIES

## II. THE UTILIZATION OF VITAMIN A ALCOHOL, VITAMIN A ACETATE AND VITAMIN A NATURAL ESTERS BY THE RAT

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In the first paper of this series (Week and Sevigne, '49) it was shown that factors affecting hydrolysis play a significant part in determining the utilization of vitamin A esters by chicks. It was also shown that in general the relative utilization of differing forms of vitamin A is radically influenced by the quantity and nature of the diluent in which the vitamin is fed.

Although it was not to be expected that rats would demonstrate the effect of hydrolytic factors to the same degree as chicks, we chose to use rats for the experiments described herein because of their wide employment in the biological appraisal of vitamin A. The importance of factors which might affect the relative utilization of different forms of the vitamin was obvious, particularly when considered in relation to the U.S.P. biological assay for vitamin A.

### EXPERIMENTAL

Weanling rats were reared on the U.S.P. XIII vitamin A assay basal diet with the addition of 1.5 units of vitamin A per 10 gm of feed. The animals were housed in screen-bottom cages under uniform conditions of light, temperature, and so forth. When the rats reached 38 days of age the fat content of the diet was increased to 16% and the animals were maintained on this high fat ration — consisting of 20% casein, 52% corn-

starch, 8% brewers' yeast, 4% salt mixture, 16% hydrogenated vegetable oil, and 60 units of vitamin D and 1.5 units of vitamin A per 10 gm of ration — until they were 52 days of age. At this time they were divided into comparable groups of 10 each with respect to sex, weight and litter.

The rats were given oral supplements by means of a calibrated tuberculin syringe of either 0.1 ml or 0.4 ml of the respective vitamin A supplement daily for three successive days. Each rat received a total of 9,000 units of vitamin A for the three-day period. The rats were killed on the 4th day, or 72 hours after receiving the first supplement, and the livers were removed and stored under refrigeration until assayed for vitamin A. Control analyses indicated no loss of vitamin A during the period of storage.

The liver of each rat was assayed for vitamin A by a modification of the procedure developed by Glover et al. ('47). Vitamin A values were determined by the Carr-Price reaction using an Evelyn colorimeter standardized with the U.S.P. Vitamin A Reference Oil. These procedures are fully described elsewhere (Week and Sevigne, '49).

The vitamin A alcohol, acetate and natural ester concentrates were prepared using a male soupfin shark liver oil as the source of vitamin A. The natural ester concentrate was made by a process which does not involve the use of high vacuum distillation. The spectrophotometric and chemical characteristics of the concentrates used in this experiment fall within the limits for preparations previously described (Week and Sevigne, '49).

The dilutions of vitamin A in corn oil, jojoba seed oil and castor oil were made on the basis of vitamin A spectrophotometric units ( $E_{1\text{ cm}}^{1\%}$ , 328 m $\mu$   $\times$  2,000) and contained 0.5% mixed tocopherols by weight.

#### DISCUSSION OF RESULTS

The analytical results are tabulated in table 1. These data were subjected to analyses of variance in order to determine the significance of the values shown.



TABLE 1

*Relationship between chemical forms of vitamin A and diluent oils, as measured by liver storage by the rat<sup>1</sup>*

DILUENT	CORN OIL			CASTOR OIL			JOJOBA SEED OIL			CORN OIL		
	0.4 ml @ 7,500 units/ml			0.4 ml @ 7,500 units/ml			0.4 ml @ 7,500 units/ml			0.1 ml @ 30,000 units/ml		
Vol. and potency of suppl. fed daily												
Total units fed in 3-day period	9,000			9,000			9,000			9,000		
Group no.	1	2	3	4	5	6	7	8	9	10	11	12
Form of vitamin A	Alcohol Acetate Ester			Alcohol Acetate Ester			Alcohol Acetate Ester			Alcohol Acetate Ester		
<i>Sex</i>	<i>Rat no.</i>											
Male	3,350	3,070	2,910	2,440	2,560	2,670	2,500	2,540	2,090	3,290	3,520	3,080
Male	2,550	2,300	2,340	2,410	2,050	2,350	2,800	2,660	1,490	3,290	3,380	2,870
Male	2,870	3,200	2,850	2,450	3,100	3,260	2,480	2,890	1,830	2,650	3,400	3,350
Male	3,100	3,610	2,310	2,890	2,860	2,910	2,780	1,930	1,800	2,480	3,550	3,370
Male	2,890	3,390	2,940	2,710	2,490	2,460	2,580	2,350	1,930	3,740	3,600	3,150
Male	2,710	3,480	3,160	2,840	3,200	3,060	2,990	2,530	1,700	2,390	3,820	2,740
Male	3,000	3,150	2,080	2,720	2,590	2,500	2,560	2,900	2,250	3,010	4,000	2,820
Female	3,060	3,580	1,880	2,980	2,610	2,430	2,840	2,460	1,980	3,160	2,560	3,810
Female	3,380	4,100	2,780	3,060	3,280	2,190	2,460	3,190	1,430	2,970	3,910	2,880
Female	2,950	2,630	3,050	2,680	2,620	3,070	2,580	2,610	1,200	2,230	3,520	3,340
Average vitamin A units per liver	2,986	3,251	2,630	2,718	2,736	2,690	2,657	2,606	1,770	2,921	3,526	3,141
Average vitamin A stored (%)	33.2	36.1	29.2	30.2	30.4	29.9	29.5	28.9	19.7	32.4	39.2	34.9

<sup>1</sup> A difference of 298 units is required for significance (5% level) between any two of test groups 1 to 9, inclusive.

A difference of 379 units is required for significance (5% level) between any two of the following test groups: 1, 2, 3, 10, 11 and 12.

Control groups, receiving diluent oils only as a supplement, showed no liver storage of vitamin A.

In the case of groups fed the 0.4 ml supplement, it is possible to make 36 separate comparisons of two groups taken at a time. Seventeen of these comparisons disclose statistically significant differences, of which the following are considered to be of interest: groups 1 with 3 and 7; 2 with 3, 5 and 8; 3 with 9; 6 with 9; 7 with 9, and 8 with 9. Certain of the comparisons which do not disclose significant differences are also of interest: groups 1 with 2 and 4; 3 with 6; 4 with 5, 6 and 7; 5 with 6 and 8, and 7 with 8.

TABLE 2

*Analyses of variance of vitamin A liver storage data listed in table 1*

SOURCE OF VARIATIONS	DEGREES OF FREEDOM	MEAN SQUARE	VARIANCE RATIO
(1) Groups 1 to 9, inclusive			
Carrier (diluent)	2	2,850,468	25.54
Individuals	9	210,583	1.89
Form of vitamin A	2	2,186,234	19.59
Carrier $\times$ form of vitamin A	4	638,253	5.72
Pooled error	72	111,606	1.00
(2) Groups 1, 2, 3, 10, 11 and 12			
Form of vitamin A	2	1,498,921	8.38
Volume of supplement	1	864,018	7.74
Pooled error	56	178,848	1.00

The comparison of group 1 with group 3 shows that factors affecting hydrolysis are of considerable importance in the utilization of vitamin A by the rat. The significantly lesser utilization of vitamin A esters versus vitamin A alcohol cannot be due to a difference of absorption per se, as the character and quantity of diluent are identical; it cannot be due to a difference of stability *in vivo*, as esters have been demonstrated to be at least equal to the free form of vitamin A in this respect; the difference must therefore be due to the effect of hydrolytic factors.

The significant difference in utilization observed in comparing group 1 with group 7 is due to the adverse effect of the

jojoba oil versus the corn oil on stability *in vivo* or absorption per se or both, in the rat. The degree to which the difference between group 3 and group 9 exceeds the difference between group 1 and group 7 demonstrates, furthermore, that the jojoba oil diluent has a significantly adverse effect, in comparison to corn oil, on the hydrolysis of vitamin A esters by the rat.

The equal utilization of the three forms of the vitamin from castor oil is interesting.

The differences in the effects of diluent variations on vitamin A acetate as compared to the effects of these variations on vitamin A natural esters are of great practical importance. These are the forms most often compared in the U.S.P. bioassay using the current U.S.P. Vitamin A Standard (vitamin A acetate). Comparisons of groups 8 with 9 and groups 5 with 6 are equally valid with respect to demonstrating the relative biological values of the two concentrates. Obviously, each comparison provides information as to relative biological efficiency, but information valid only for the specific conditions of the experiment in question.

In the cases of groups 1, 2, 3, 10, 11 and 12, it is possible to make 15 comparisons of two groups at a time. Of these, 6 disclose statistically significant differences, of which the following are considered to be important: groups 2 with 3, 3 with 12, 10 with 11, and 11 with 12.

The improved utilization of the ester forms in the smaller quantity of corn oil diluent is most interesting. The U.S.P. bioassay is conducted with a daily feeding of not more than 0.1 ml of diluent oil. The significant difference observed in vitamin A ester utilization in comparing group 3 with group 12 is due to the effect of the volume of corn oil on hydrolysis *in vivo*, as the change in volume has negligible effect on absorption per se or stability *in vivo* or both (compare group 1 with group 10).

#### REMARKS

The results of these investigations show that in addition to the stability factors described by Hickman et al. ('44), and others, as affecting the relative biological response of differing

forms of vitamin A, factors influencing the hydrolysis *in vivo* of vitamin A esters may be of equal or greater significance. While the experimental results of the present study do not afford a direct quantitative measure of the factors which affect the hydrolysis of vitamin A esters under conditions of normal or sub-minimum intake of the vitamin, the data suggest certain implications regarding the experimental methods now employed in vitamin A biological assays. These methods of bioassay, namely the U.S.P. rat growth test and the Guggenheim and Koch ('44) liver storage technique, make comparisons between dissimilar chemical forms of vitamin A under one arbitrary set of experimental conditions. Our results show that certain modifications of these conditions, such as variations in the nature or quantity of the diluent used for the vitamin A supplements, radically affect the relative biological efficacy of the different chemical forms of vitamin A.

No single set of arbitrary test conditions can be shown to be more valid than another for the purpose of appraising the relative biological efficiency of differing forms of vitamin A under the multifold conditions of final use. This being true, a biological assay intended for quantitative determinations should be designed simply to measure the mol quantities of biologically active vitamin A present in test samples. If current biological assay methods were to be modified to meet this requirement, better agreement could be attained between physico-chemical and biological measurements of vitamin A, and agreement between laboratories on biological assays would be greatly improved.

As the simplest means of adapting biological assays to this desirable end, we suggest that both test sample and the Reference Standard be saponified, and that the biological test be conducted using the unsaponifiable extracts. By this method, the various esters of vitamin A are converted to a single chemical form, i.e., vitamin A alcohol; and variations in diet and diluent which may affect the stability, hydrolysis and absorption of the vitamin act in parallel fashion on both sample and Standard.

## SUMMARY

1. Vitamin A liver storage measurements were used as the criterion for determining the relative utilization by the rat of the alcohol, acetate and natural ester forms of vitamin A under various experimental conditions.

2. It was shown that the relative biological efficacy of the three forms of the vitamin, although administered at equivalent unit levels, was greatly affected by the nature and quantity of the diluent present in the vitamin A supplement. Experimental conditions were established under which the relative biological response produced by the natural ester form could be varied from 59 to 105% of that produced by comparable supplements of vitamin A alcohol. It was also demonstrated that variations in the biological utilization of vitamin A acetate under diverse experimental conditions are significantly different from those of vitamin A natural esters.

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# PROTECTIVE EFFECTS OF SOYBEAN MEAL FOR THE IMMATURE HYPERTHYROID RAT<sup>1</sup>

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Available data indicate that requirements for a number of nutrients are markedly increased in the hyperthyroid animal (Drill, '43). This is particularly true for some of the B vitamins. An increased requirement for thiamine (Drill, '38; Drill and Sherwood, '38), pyridoxine and pantothenic acid (Drill and Overman, '42), folic acid (Martin, '47) and, more recently, vitamin B<sub>12</sub> (Nichol et al., '49; Emerson, '49; Bethel and Lardy, '49) has been demonstrated following the administration of large doses of thyroactive substances. Requirements are also increased for various unknown factors as well. These "minor vitamins" are apparently dispensable under normal conditions, or requirements for them are so small they may readily be met by amounts present in the diet or through the synthetic activity of the intestinal flora or the animal's own tissues. During hyperthyroidism, however, requirements for these substances may be increased to the extent that deficiencies occur, manifested by retarded growth or tissue pathology and preventable by the administration in appropriate amounts

<sup>1</sup> This paper reports research undertaken in cooperation with the Quartermaster Food and Container Institute for the Armed Forces, and has been assigned number 245 in the series of papers approved for publication. The views or conclusions contained in this report are those of the author. They are not to be construed as necessarily reflecting the views or indorsement of the Department of the Army.

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of the missing nutrient (Ershoff, '48a). Thus, yeast has been found to prolong significantly the average survival time of immature rats fed purified rations containing massive doses of thyroid (Ershoff and Hersberg, '45). Similar effects have been noted with desiccated and defatted whole liver (Ershoff, '47a; Bethel et al., '47). The latter material was also found to counteract completely the retardation in growth (Ershoff, '47a, '47b; Bethel et al., '47) and inhibition of ovarian development (Ershoff, '47b) of young rats fed massive doses of thyroid. Some confusion exists, however, regarding the nature of the factor or factors in liver responsible for the above effects. On rations containing soybean meal the retardation in growth caused by massive doses of desiccated thyroid or iodinated casein has been counteracted both in the rat and chick by crystalline vitamin B<sub>12</sub> (Nichol et al., '49; Emerson, '49). On rations containing casein as the protein and sucrose as the carbohydrate, however, the retardation in growth following administration of massive doses of thyroactive substances appears to be due, at least in part, to a deficiency of some other factor (Ershoff, '48b, '49; Bethel and Lardy, '49), which has been termed the "antithyrotoxic factor of liver." It is present in considerable concentration in the water-insoluble fraction of liver (Ershoff, '47a, '48b; Ershoff and McWilliams, '48) and is distinct from any of the known nutrients, including vitamin B<sub>12</sub> (Ershoff, '49). In view of the different deficiencies which are produced by thyroid feeding in animals fed the above rations, the following experiments were undertaken in an effort to determine the factor or factors in soybean meal responsible for the diverse results.

All experiments were performed with female rats of the Long-Evans strain, from 23 to 25 days of age, housed in metal cages with raised screen bottoms to minimize access to the feces. After being fed the respective experimental diets ad libitum for a period of 28 days the animals were sacrificed and the weights of the thyroids, adrenals, ovaries, kidneys and heart ventricles determined.



## EXPERIMENTAL

*Experiment no. 1. On the antithyrototoxic properties of a full-fat soybean meal<sup>2</sup>*

Two basal rations were employed in this experiment, diet A and diet B (table 1). Diet A was a purified ration containing casein as the dietary protein. Diet B was similar in composition but contained a full-fat soybean meal. Each of the above diets was supplemented with U.S.P. desiccated thyroid<sup>3</sup> or iodinated casein<sup>4</sup> added in place of an equal amount of sucrose. Thyroid was incorporated in the basal rations at levels of 0.25% and 0.5%; iodinated casein at a level of 0.125%. Seventy-

TABLE 1  
*Composition of experimental diets*

DIETARY COMPONENT	DIET A	DIET B
	%	%
Casein <sup>1</sup>	22.0	5.0
Soybean meal <sup>2</sup>	0.0	45.0
Salt mixture <sup>3</sup>	4.5	4.5
Sucrose	73.5	45.5

Vitamin supplements: to each kilogram of the above diets were added the following synthetic vitamins: thiamine hydrochloride 72 mg, riboflavin 9 mg, pyridoxine hydrochloride 15 mg, calcium pantothenate 67.2 mg, nicotinic acid 60 mg, 2-methyl-naphthoquinone 5 mg, and choline chloride 1.2 gm.<sup>4</sup> Each rat also received three times weekly the following supplement: cottonseed oil (Wesson) 500 mg, alpha-tocopherol acetate 1.5 mg, and a vitamin A-D concentrate<sup>5</sup> containing 50 U.S.P. units of vitamin A and 5 U.S.P. units of vitamin D.

<sup>1</sup> Vitamin test casein, General Biochemicals, Inc., Chagrin Falls, Ohio.

<sup>2</sup> Soya meal, El Molino Mills, Alhambra, California, a lightly roasted material containing 18 to 22% fat.

<sup>3</sup> Salt mixture no. 1 (Sure, '41).

<sup>4</sup> In view of the increased requirements for thiamine, pyridoxine and pantothenic acid in the hyperthyroid rat (Drill and Overman, '42), the B vitamins in the present experiment were administered in excessive amounts in order to assure an adequacy of these factors in the diet.

<sup>5</sup> Nopco fish oil concentrate, assaying 800,000 U.S.P. units of vitamin A and 80,000 U.S.P. units of vitamin D per gram.

<sup>2</sup> The term "full-fat soybean meal" as employed in the present communication refers to soybean meal with an average fat content of 18 to 22%.

<sup>3</sup> Thyroid powder, U.S.P., Armour and Co., Chicago, Ill.

<sup>4</sup> Protamone, Cerophyl Laboratories, Kansas City, Missouri.

six female rats of the Long-Evans strain were selected for the present experiment. Their average weight was 46.2 gm.

Data on growth are summarized in table 2. These data indicate that the retardation in growth caused by massive doses of desiccated thyroid or iodinated casein on diet A did not occur on diet B. On the latter ration no significant difference was observed between the gain in body weight of animals fed the control ration and that of those fed a similar diet supple-

TABLE 2

*Effects of full-fat soybean meal on the gain in body weight of immature rats fed massive doses of desiccated thyroid and iodinated casein*

The values in parentheses indicate the number of animals which survived and on which averages are based

THYROACTIVE SUPPLEMENT	NUMBER OF RATS		INITIAL BODY WT.		GAIN IN BODY WT. OVER 28-DAY PERIOD <sup>1</sup>	
	Diet A	Diet B	Diet A	Diet B	Diet A	Diet B
			gm	gm	gm	gm
None	8	8	46.1	46.0	100.8 ± 3.3 (8)	102.3 ± 4.1 (8)
0.25% thyroid	10	10	45.6	46.0	60.9 ± 6.5 (9)	107.3 ± 5.1 (10)
0.5% thyroid	10	10	46.6	46.1	45.4 ± 6.0 (8)	104.5 ± 3.3 (10)
0.125% iodinated casein	10	10	46.4	47.0	59.4 ± 4.4 (9)	102.8 ± 5.4 (9)

<sup>1</sup> Including the standard error of the mean calculated as follows  $\sqrt{\frac{\sum d^2}{n}} / \sqrt{n}$ , where "d" is the deviation from the mean and "n" is the number of observations.

mented with desiccated thyroid or iodinated casein. Data on organ weights are summarized in table 3. The findings indicate that adrenal, kidney and ventricular weights, when expressed in terms of organ weight per 100 gm body weight, were increased and thyroid weight was decreased on both diets A and B in animals fed desiccated thyroid or iodinated casein. The relative increase in adrenal, kidney and ventricular weights in animals fed thyroid or iodinated casein was greater

TABLE 3

*Effects of full fat soybean meal on organ weights of immature rats fed massive doses of desiccated thyroid and iodinated casein*

Dietary Group	Thyroid activity supplement	Body wt. after 28 days of feeding	Ovarian wt. <sup>1</sup>	Thyroid wt.	Thyroid mg/100 gm body wt.	Adrenal wt.	Adrenal mg/100 gm body wt.	Enteric ulcer wt.	Enteric ulcer mg/100 gm body wt.	Adrenal y wt.	Adrenal y mg/100 gm body wt.
		gm	mg	mg		mg		mg		µm	
A	None	146.9	42.8 ± 2.5	9.5	0.5	35.8	24.1	564	381	1.575	1.072
A	0.25% thyroid	107.8	25.3 ± 2.7	5.9	5.5	36.3	33.7	809	750	2.041	1.893
A	0.5% thyroid	92.7	22.7 ± 2.9	5.5	5.9	29.0	31.3	739	797	2.137	2.305
A	0.125% iodinated casein	105.1	20.0 ± 1.5	6.1	5.8	31.2	29.6	811	769	2.039	1.935
B	None	148.3	43.1 ± 3.2	9.9	6.7	37.7	25.4	513	366	1.573	1.061
B	0.25% thyroid	153.3	40.7 ± 6.0	8.8	5.7	43.9	28.6	729	176	2.188	1.427
B	0.5% thyroid	150.6	28.8 ± 2.6	8.3	5.5	42.3	28.1	767	509	2.234	1.180
B	0.125% iodinated casein	150.8	38.8 ± 6.2	8.7	5.8	43.9	29.1	750	497	2.113	1.421

<sup>1</sup> Including the standard error of the mean. See footnote 1, table 2.

on diet A than on diet B. Ovaries appeared immature both in weight and microscopic appearance in animals fed diet A supplemented with desiccated thyroid or iodinated casein. On diet B, however, 7 out of 10 rats fed thyroid at a level of 0.25% and 6 out of 9 animals fed iodinated casein had apparently normal ovaries. When thyroid was fed at a level of 0.5%, only two out of 10 rats had ovaries which appeared normal. No abnormalities in weight or microscopic appearance were observed in the ovaries of rats fed similar rations with thyroid or iodinated casein omitted. The protective effect of full-fat soybean meal was also evident in respect to gross appearance. On diet A animals fed thyroid or iodinated casein had an unthrifty appearance, with ruffled fur and occasionally alopecia; on diet B, however, animals fed thyroid or iodinated casein were indistinguishable grossly from the normal controls.

Basal metabolic rates were determined on the 25th day of the experiment for animals fed the various soybean rations.<sup>5</sup> No attempt was made to obtain readings for animals fed diet A supplemented with thyroid or iodinated casein in view of the high susceptibility of such animals to heart failure following excitation. Previous findings from this laboratory indicate that rats maintained on synthetic rations supplemented with massive doses of thyroid readily succumb following unaccustomed handling. In preliminary tests, 7 out of 8 rats fed diet A supplemented with 0.5% thyroid died in the respiration chamber during the course of oxygen determinations; on the other hand, animals fed similar rations supplemented with 10% whole liver or 10% yeast survived similar handling (Ershoff, '47a).

Eight rats in each experimental group were tested. The apparatus used was of a closed circuit type, with a capacity of 2 l (Mason, Kryder and Winzler, '49). Carbon dioxide was absorbed with sodium hydroxide, and oxygen consumption was determined from pressure changes recorded by means of a

<sup>5</sup> We are indebted to Mr. George Kryder of the Department of Biochemistry and Nutrition, University of Southern California, for the determinations of basal metabolic rates.

water manometer. The respiration chambers were kept at 27°C. and readings obtained were corrected to standard temperature and pressure. Food was removed from the animals' cages the evening prior to the metabolism test. At least 6 successive 5-minute intervals were recorded for each animal, with care being taken to measure oxygen consumption when animal activity was at a minimum. The two lowest readings were employed in calculating the basal rate. Results are summarized in table 4. They indicate that full-fat soybean meal did not counteract the rise in basal metabolic rate caused by

TABLE 4

*Effects of desiccated thyroid and iodinated casein on the basal metabolism of immature rats fed diets containing full-fat soybean meal*

DIETARY GROUP	THYROIDACTIVE SUPPLEMENT	NUMBER OF ANIMALS	O <sub>2</sub> CONSUMPTION ML/HR./100 GM BODY WT. <sup>1,2</sup>
A	None	8	139.0 ± 2.4
B	None	8	133.7 ± 3.4
B	0.25% thyroid	8	227.4 ± 4.3
B	0.5% thyroid	8	246.2 ± 6.4
B	0.125% iodinated casein	7 <sup>3</sup>	229.9 ± 8.2

<sup>1</sup> Including the standard error of the mean. See footnote 1, table 2.

<sup>2</sup> This method of reporting these data is used, rather than "0.75 power of the body weight" or some similar expression, inasmuch as there was substantially little difference in the body weights of the animals.

<sup>3</sup> Originally 8 rats were present in this group; one animal died during the determination.

desiccated thyroid or iodinated casein. It is apparent, therefore, that the protective effect of soybean meal as observed in the present experiments was an antithyrototoxic effect, and was not due to neutralization, destruction or impaired absorption of the thyroid hormone.

The presence of a goitrogenic agent in soybeans has been demonstrated by McCarrison ('33) and confirmed by Sharpless ('38) and others (Sharpless et al., '39). Thyroid enlargement was demonstrated by these workers only on low iodine diets, since the goitrogenic effects of soybean were counteracted by iodine (Sharpless et al., '39). In the present experi-

ment no goitrogenic effects such as increase in thyroid weight or reduction in basal metabolic rate resulted from the feeding of full-fat soybean meal, a finding presumably due to the high iodine content of the diet employed.

*Experiment no. 2. Comparative effects of raw and autoclaved full-fat soybean meal on the growth of immature thyroid-fed rats*

The following experiment was undertaken to determine the effects of autoclaving on the growth-promoting properties of

TABLE 5

*Comparative effects of raw and autoclaved full-fat soybean meal on the gains in body weight of immature rats fed massive doses of desiccated thyroid*

The values in parentheses indicate the number of animals which survived and on which averages are based

GROUP	NUMBER OF ANIMALS	INITIAL BODY WT.	GAIN IN BODY WT. AFTER 28 DAYS OF FEEDING <sup>1</sup>
I	6	gm 44.6	gm 99.8 ± 7.1 (6)
II	8	44.7	96.8 ± 4.4 (7)
III	8	44.5	93.3 ± 4.4 (7)

<sup>1</sup> Including the standard error of the mean. See footnote 1, table 2.

full-fat soybean meal for the immature thyroid-fed rat. Three experimental groups were employed. Group I was fed diet B (table 1); group II received diet B plus 0.5% desiccated thyroid<sup>6</sup> (with thyroid added in place of an equal amount of sucrose); group III was fed a diet similar to that fed group II but containing soybean meal which had been autoclaved for 30 minutes at a pressure of 15 pounds. Twenty-two female rats with an average weight of 44.6 gm were selected for the present experiment. The results are summarized in table 5. No significant differences in gain in body weight were observed for

<sup>6</sup> See footnote 3, page 261.

animals fed any of the diets employed. It is apparent, therefore, that autoclaving under the conditions of the present experiment did not impair the growth-promoting effect of full-fat soybean meal for the immature thyroid-fed rat.

*Experiment no. 3. Comparative effects of low-fat soybean flour and full-fat soybean meal on the growth of immature thyroid-fed rats*

This experiment was undertaken to determine the comparative effects of low fat soybean flour and full-fat soybean meal on the gains in body weight of immature rats fed massive doses of desiccated thyroid. Three soybean sources were employed: (1) a full-fat soybean meal;<sup>7</sup> (2) an extracted soybean flour containing 4% fat;<sup>8</sup> and (3) an expeller-processed soybean flour containing 7% fat.<sup>9</sup> The rations fed were similar to diet B (table 1) but differed in the source of the soybean product employed. Diets were fed with and without desiccated thyroid,<sup>10</sup> which was administered at a level of 0.5% in place of an equal amount of sucrose. Forty-eight female rats with an average weight of 46.0 gm were selected for this experiment. The results are summarized in table 6.

The findings indicate that a direct correlation exists between the fat content of the soybean component of the diet and its antithyrototoxic effect. The gain in body weight of immature rats fed massive doses of desiccated thyroid was greatest on a diet containing full-fat soybean meal (18 to 22% fat) and least on a ration containing extracted soybean flour (4% fat). Values for the diet containing "expeller" soybean flour (7% fat) lay between these two. When thyroid was omitted from the above rations, no significant difference in growth was observed on any of the diets employed. It would appear, therefore, that the antithyrototoxic properties of full-fat soybean meal were

<sup>7</sup> Soya meal, El Molino Mills, Alhambra, Calif. This material was similar to that employed in experiments 1 and 2 but came from another lot.

<sup>8</sup> Extracted soy flour (4% blended), A. E. Staley Mfg. Co., Decatur, Ill.

<sup>9</sup> Soy Flour Lo-Fat, A. E. Staley Mfg. Co., Decatur, Ill.

<sup>10</sup> See footnote 3, page 261.

correlated with its fat content, and that soybean oil or some factor in it was responsible for the antithyrototoxic effect.

*Experiment no. 4. Comparative effects of vitamin B<sub>12</sub>,  
extracted liver residue and soybean oil on the  
gain in body weight of immature rats fed  
massive doses of desiccated thyroid*

Previous findings indicate that vitamin B<sub>12</sub> counteracts the growth-retarding effect of massive doses of thyroid when fed

TABLE 6

*Comparative effects of low fat soybean flour and full-fat soybean meal on gain in  
body weight of immature rats fed massive doses of desiccated thyroid*

The values in parentheses indicate the number of animals which survived and on which averages are based

SOYBEAN COMPONENT IN DIET	THYROID	NUMBER OF ANIMALS	INITIAL BODY WT.	GAIN IN BODY WT. AFTER 28 DAYS OF FEEDING <sup>1</sup>
	%		gm	gm
Extracted soybean flour	0.5	10	45.9	56.5 ± 4.6
4% fat				(8)
"Expeller" soybean flour	0.5	10	46.0	70.4 ± 3.6
7% fat				(10)
Full-fat soybean meal	0.5	10	46.1	97.1 ± 4.8
18-22% fat				(8)
Extracted soybean flour	0.0	6	45.8	88.0 ± 5.1
4% fat				(6)
"Expeller" soybean flour	0.0	6	46.0	87.4 ± 3.6
7% fat				(6)
Full-fat soybean meal	0.0	6	46.0	99.2 ± 3.7
18-22% fat				(6)

<sup>1</sup> Including the standard error of the mean. See footnote 1, table 2.

in conjunction with a diet containing soybean meal (Emerson, '49). The present experiment was undertaken to determine the comparative effects of vitamin B<sub>12</sub> and extracted liver on the gain in body weight of immature rats fed massive doses of thyroid in conjunction with diets containing either casein or low-fat soybean flour as the principal source of protein. In view of the experiments reported above, in which full-fat soy-



bean meal exerted an antithyrototoxic effect, animals on each of the above rations were also fed soybean oil (at a level corresponding to its content in diets containing full-fat soybean meal) in an effort to determine what effect this material might have on the gain in body weight of immature thyroid-fed rats.

Two basal rations were employed, diet A and diet C. Diet A was a purified ration containing casein as the dietary protein; diet C was similar in composition but contained a low fat soybean flour.<sup>11</sup> These diets were identical with diets A and B (table 1), except that a low fat soybean flour replaced the full-fat soybean meal of diet B. Five experimental groups were fed each of the basal rations. Group I was fed the basal ration alone; group II the basal ration plus 0.5% desiccated thyroid;<sup>12</sup> group III the basal ration plus 0.5% desiccated thyroid plus 30  $\mu$ g vitamin B<sub>12</sub> per kilogram of diet;<sup>13</sup> group IV the basal ration plus 0.5% desiccated thyroid plus 10% extracted liver residue;<sup>14</sup> and group V the basal ration plus 0.5% desiccated thyroid plus 10% cold pressed soybean oil. The dietary supplements and the desiccated thyroid were added in place of an equal amount of sucrose.

Eighty female rats with an average weight of 44.8 gm were selected for this experiment. The effects of the various diets on body and ovarian weights are summarized in table 7.

The findings indicate that animals fed diet A differed significantly from those fed diet C following the administration of massive doses of thyroid. On diet A the retardation in growth of thyroid-fed rats (group II) was completely counteracted by extracted liver residue (group IV), partially counteracted by soybean oil (group V), and unaffected by vitamin B<sub>12</sub> (group III). On diet C the growth retardation of thyroid-fed rats (group II) was completely counteracted by all three sup-

<sup>11</sup> See footnote 9, page 267.

<sup>12</sup> See footnote 3, page 261.

<sup>13</sup> The vitamin B<sub>12</sub> employed in the present experiment was obtained from Merek and Co., Rahway, N. J.

<sup>14</sup> Extracted liver residue, Wilson Laboratories, Chicago, Ill. This product consists of the coagulated, water-insoluble material remaining after the removal of the extractable water-soluble substances.

TABLE 7

*Comparative effects of vitamin B<sub>12</sub>, extracted liver residue and soybean oil on body and ovarian weights of immature rats fed massive doses of desiccated thyroid*

The values in parentheses indicate the number of animals which survived and on which averages are based

DIETARY SUPPLEMENT	THYROID %	NUMBER OF RATS		INITIAL BODY WT.		GAIN IN BODY WEIGHT OVER 28-DAY PERIOD <sup>1</sup>		OVARIAN WT. <sup>1</sup>	
		Diet A	Diet C	Diet A	Diet C	Diet A	Diet C	Diet A	Diet C
None	0.5	8	8	gm 44.9	gm 45.0	gm 48.4 ± 3.9 (5)	gm 68.3 ± 3.0 (6)	mg 24.0 ± 2.2	mg 25.9 ± 2.3
Vitamin B <sub>12</sub>	0.5	8	8	44.7	45.0	51.8 ± 3.6 (6)	93.4 ± 4.1 (8)	25.1 ± 2.8	35.6 ± 4.1
Extracted liver residue	0.5	8	8	44.4	44.8	102.6 ± 4.0 (6)	99.0 ± 5.1 (7)	48.8 ± 6.1	28.7 ± 3.2
Soybean oil	0.5	8	8	45.0	45.0	78.4 ± 4.3 (7)	92.6 ± 3.7 (7)	27.8 ± 2.6	29.5 ± 3.3
None	0.0	8	8	44.4	44.6	99.2 ± 4.7 (8)	91.2 ± 4.6 (8)	43.1 ± 2.7	39.8 ± 3.9

<sup>1</sup> Including the standard error of the mean. See footnote 1, table 2.

plements (groups III, IV and V). Furthermore, thyroid-fed rats on diet C gained significantly more weight than similar animals (group II) on diet A. In the absence of dietary thyroid (group I), no significant differences in growth were observed between rats fed either of the basal diets.

Differences in ovarian weights were noted between rats fed diets A and C. On diet A ovaries appeared immature both in weight and microscopic appearance in groups II, III and V. Among the thyroid-fed rats, only those fed extracted liver residue (group IV) had ovaries which were similar in weight and in histological appearance to those of the normal controls (group I). On diet C, however, ovarian weight was retarded in all thyroid-fed rats (groups II, III, IV and V). Kidney, adrenal and ventricular weights were increased on both diets A and C in all thyroid-fed rats.

*Experiment no. 5. Comparative effects of soybean oil and other vegetable and animal fats on the gains in body weight of immature rats fed massive doses of desiccated thyroid*

The basal ration employed in this experiment was diet A (table 1). Animals were fed diet A plus 0.5% desiccated thyroid<sup>15</sup> plus 10% of each of the following fats: olive oil, peanut oil, cottonseed oil, wheat germ oil, corn oil, soybean oil, hydrogenated cottonseed oil<sup>16</sup> and lard.<sup>17</sup> Control groups were fed diet A alone and diet A plus 0.5% desiccated thyroid. Thyroid and the various fats were incorporated in the above diets in place of an equal amount of sucrose.

<sup>15</sup> See footnote 3, page 261.

<sup>16</sup> Crisco.

<sup>17</sup> The fats were obtained from the following sources: olive oil, Strohmeyer and Arpe Co., New York, N. Y.; peanut oil, Planters Edible Oil Co., San Francisco, Calif.; cottonseed oil, Wesson Oil and Snowdrift Sales Co., New Orleans, La.; wheat germ oil, VioBin Corporation, Monticello, Ill.; corn oil, Corn Products Refining Co., Argo, Ill.; soybean oil, House of Better Living, Los Angeles, Calif.; Crisco, Procter and Gamble, Cincinnati, Ohio; and lard, Rath Packing Co., Waterloo, Iowa.

Eighty-seven female rats with an average weight of 45.9 gm were selected for this experiment. Diets were prepared bi-weekly and stored under refrigeration.

The effects of the various diets on gain in body weight are summarized in table 8. These indicate that massive doses of

TABLE 8

*Comparative effects of vegetable and animal fats on the gains in body weight of immature rats fed massive doses of desiccated thyroid*

The values in parentheses indicate the number of animals that survived and on which averages are based

SUPPLEMENT ADDED TO BASAL RATION	THYROID	NUMBER OF ANIMALS	INITIAL BODY WT.	GAIN IN BODY WT. OVER 28-DAY PERIOD <sup>1</sup>
	%		gm	gm
None	0.5	15	46.0	44.7 $\pm$ 2.9 (9)
Olive oil	0.5	8	46.1	67.7 $\pm$ 1.6 (6)
Peanut oil	0.5	8	45.6	69.6 $\pm$ 1.4 (6)
Cottonseed oil	0.5	8	45.9	70.1 $\pm$ 4.1 (7)
Wheat germ oil	0.5	8	45.8	73.8 $\pm$ 3.0 (5)
Corn oil	0.5	8	45.8	74.6 $\pm$ 3.9 (7)
Soybean oil	0.5	8	46.1	74.7 $\pm$ 3.5 (7)
Crisco	0.5	8	45.9	76.2 $\pm$ 4.5 (6)
Lard	0.5	8	46.0	82.9 $\pm$ 3.9 (5)
None	0.0	8	45.4	101.8 $\pm$ 5.7 (8)

<sup>1</sup> Including the standard error of the mean. See footnote 1, table 2.

thyroid caused a marked retardation in the gains in body weight of immature rats fed a low fat diet. The growth-retarding effect of thyroid was partially counteracted by the increased administration of fat. All fats tested increased growth significantly over that observed in thyroid-fed rats on the low

fat diet, but gain in body weight was most marked for the animals fed lard. In view of the small number of rats employed, it is doubtful if the differences in growth among animals fed the various fat-containing diets were statistically significant. Although increasing the fat content of the diet augmented the gains in body weight of immature rats fed massive doses of thyroid, it did not counteract other effects of thyroid administration, such as inhibition of ovarian development or increase in kidney, adrenal and ventricular weights. Soybean oil was not superior to other fats in its ability to promote growth in the immature thyroid-fed rat.

#### DISCUSSION

It is becoming increasingly apparent that the effects of thyroid feeding are dependent to a considerable degree on the composition of the basal ration. It is well known that massive doses of thyroid cause a marked retardation in growth in the immature rat. Recent findings indicate, however, that the growth-retarding effect of thyroid may be completely counteracted by the administration of defatted and desiccated whole liver (Ershoff, '47a, '47b; Bethel et al., '47) and other animal tissues such as defatted and desiccated heart, kidney, placenta, duodenum, thymus and brain (Ershoff, '48b).

Massive doses of thyroid have also been found to inhibit ovarian development in the immature rat (Ershoff, '45). This effect may also be counteracted by the administration of defatted and desiccated whole liver (Ershoff, '47b). Liver feeding, however, does not prevent the rise in oxygen consumption nor the increase in adrenal, kidney and ventricular weights following thyroid feeding, nor will it prevent the attendant reduction in ventricular creatine concentration (Ershoff, '47a). It is apparent that the protective effects of liver noted above are *antithyrototoxic* effects, and not due to neutralization, elimination or destruction of the thyroid hormone.

In the present series of experiments data were obtained indicating that full-fat soybean meal also exerts an antithyrototoxic

effect in the immature female rat. The protective effects of this substance are due in part to its fat content, but there appears to be at least one factor in soybeans other than their fat content which also plays a part in counteracting symptoms of thyrotoxicity in the immature rat.

A number of reports have appeared concerning the beneficial effects of dietary fat for the hyperthyroid animal. Abelin and co-workers (Abelin, '26; Abelin and Kürsteiner, '28; Abelin, Knuchel and Spichtin, '30) observed that increasing the fat content of the diet counteracted in part the rise in basal metabolic rate and the reduction in liver glycogen following thyroid feeding. Berg ('34) found that large amounts of vegetable fats lowered the metabolic rates of hyperthyroid dogs. Similar findings have been observed with respect to certain animal and vegetable fats and the hyperthyroid rat (Munoz, '44; Spalloni Cialdea, '45; Hoffmann, Hoffmann and Talesnik, '45). Available data indicate that the protective effects of fat noted above were due, at least in part, to certain unsaturated fatty acids.

Keeser ('38) reported that sodium oleate and linoleic acid protected against the rise in oxygen consumption following administration of the thyroid hormone; while Guerra ('47) observed that the protective effects of animal and vegetable fats against the rise in basal metabolic rate following thyroid feeding were correlated with the degree of unsaturation of the various fats. Similar data concerning the protective effects of certain unsaturated fatty acids on the hyperthyroid animal have been reported by Zain ('36, '37). This investigator found that linoleic acid, but not stearic acid, would prevent reduction in liver glycogen following administration of massive doses of thyroid to the female rat. Values obtained with oleic acid lay between these two. Linoleic acid also protected against loss in weight following thyroid feeding. Although some of the effects noted above may have been due, at least in part, to an increased caloric intake or to the ingestion of fat-soluble vitamins or other nutrients present in the various fats, the data nevertheless indicate that fat per se, and certain fatty

acids in particular, may modify the effects of excessive amounts of thyroid hormone.

The results of the present experiments provide further data concerning the beneficial effects of fat on the hyperthyroid animal. It was observed that increasing the fat content of the diet partially counteracted the growth retardation of immature rats fed massive doses of thyroid in conjunction with a low fat diet. A number of fats were tested at a level of 10% in the diet for their growth-promoting effects on the immature hyperthyroid rat. Olive oil, peanut oil, cottonseed oil, wheat germ oil, corn oil, soybean oil, hydrogenated cottonseed oil and lard were all found to cause a gain in body weight significantly greater than that observed in thyroid-fed rats on a control low fat ration. They did not, however, counteract other effects of thyroid administration, such as inhibition of ovarian development or increase in kidney, adrenal and ventricular weights.

No data are available concerning the mechanism by which fat exerts its growth-promoting effect on the immature hyperthyroid rat. It is possible that requirements for certain fatty acids are increased in the hyperthyroid animal to the extent that a deficiency of these nutrients is precipitated on diets low in fat. The gain in body weight following the administration of various fats might be regarded under these circumstances as due to the correction of a nutritional deficiency. The butyl alcohol residue of extracted liver residue, however, which contains virtually no fat, is far more effective than any of the above fats in counteracting the growth retardation of hyperthyroid rats on a low fat diet (Ershoff, '48b). It would appear, therefore, that the growth-promoting effect of fat on the hyperthyroid rat is due to some factor other than the correction of a fatty acid deficiency. It is possible that fat may stimulate the synthesis of an antithyrototoxic factor by the intestinal flora. Whipple and Church's finding ('35) that lard exerts a favorable effect on the intestinal synthesis of thiamine in the rat may be pertinent in this regard. No data are available indicating to what extent differences in growth

on the various rations may have been due to differences in caloric intake.

The present experiments indicate that full-fat soybean meal completely counteracts the growth retardation of immature rats fed massive doses of desiccated thyroid. Gain in body weight was considerably reduced, however, when low fat soybean flour was fed in place of the full-fat soybean meal. When thyroid was omitted from the above rations, no significant differences in growth were observed on any of the diets employed. The retardation in growth of thyroid-fed rats on a diet containing low fat soybean flour was completely counteracted by the administration of 10% soybean oil, added in place of an equal amount of sucrose. Similar results were obtained, however, with the administration of 10% extracted liver residue or crystalline vitamin B<sub>12</sub> when fed at a level of 30 µg per kilogram of diet.

The significance of these findings is not readily apparent. Although the protective effects of extracted liver residue might conceivably be due to a bound form of vitamin B<sub>12</sub>, it is difficult to account for the equal effectiveness of soybean oil. If the assumption is made that the growth retardation of hyperthyroid rats on a diet containing low fat soybean flour is due to a vitamin B<sub>12</sub> deficiency, an interpretation in accord with previous findings (Emerson, '49), it follows that soybean oil either: (1) decreases body requirements for vitamin B<sub>12</sub>; (2) stimulates the synthesis of vitamin B<sub>12</sub> by the intestinal flora or the animal's own tissues; or (3) contains significant amounts of a substance with vitamin B<sub>12</sub> activity. No data are available for determining which of these alternatives, if any, is correct.

In agreement with earlier findings (Ershoff, '48b, '49), vitamin B<sub>12</sub> in the present experiments failed to counteract the growth retardation of hyperthyroid rats fed a diet low in fat and containing casein as the dietary protein. Animals fed extracted liver residue under similar experimental conditions, however, gained as much weight as the normal controls. These results indicate that the growth-promoting effect of



extracted liver residue is not due to its vitamin B<sub>12</sub> content. The gains in body weight of thyroid-fed rats receiving soybean oil was intermediate between those of animals fed vitamin B<sub>12</sub> and those of rats fed extracted liver residue.

Data on ovarian weight also indicate that the protective effects of extracted liver residue, under the conditions of the above experiment, were due to some factor other than vitamin B<sub>12</sub>. The ovaries of thyroid-fed rats on the vitamin B<sub>12</sub> supplement were immature both in weight and microscopic appearance. Similar findings were obtained with animals fed soybean oil. The ovaries of rats fed extracted liver residue, however, were indistinguishable grossly and in histological appearance from those of the normal controls. The protective effects of extracted liver residue were presumably due to an unidentified nutrient, which has been termed the "antithyrotoxic factor of liver." Available data indicate that this factor is distinct from any of the known nutrients, including vitamin B<sub>12</sub> (Ershoff, '47a, '48b, '49; Bethel et al., '47).

It is apparent from the above findings that massive doses of thyroid can precipitate a deficiency of either vitamin B<sub>12</sub> or the "antithyrotoxic factor of liver" in the immature female rat, depending on the composition of the basal ration. When immature female rats were fed a low fat diet containing casein as the dietary protein, the retardation in growth following thyroid feeding could be counteracted by extracted liver residue but not by vitamin B<sub>12</sub>, indicating that it was due to a deficiency of the "antithyrotoxic factor of liver." When immature female rats were fed a similar diet containing low fat soybean flour as the dietary protein, the resulting growth retardation was due to a deficiency of vitamin B<sub>12</sub>. The question immediately arises of why animals fed the low fat soybean flour did not develop a deficiency of the "antithyrotoxic factor of liver" and, conversely, why rats fed the casein-containing ration did not develop a deficiency of vitamin B<sub>12</sub>.

It was observed in the course of the present experiments that when immature female rats were fed massive doses of thyroid in conjunction with a low fat diet, they gained signifi-

cantly more weight on a ration containing low fat soybean flour as the dietary protein than they did on a diet containing purified casein. Since the amount of thyroid in both diets was identical, it is apparent that the soybean flour either: (1) contained one or more factors which counteracted in part the growth-retarding effects of thyroid; or (2) stimulated the synthesis of such a factor or factors by the intestinal flora or the animal's own tissues.

Although part of the growth-promoting effect of low fat soybean flour may have been due to its fat content (7%), it would appear that it contains at least one additional factor which is qualitatively similar to, if not identical with, the "antithyrototoxic factor of liver." The failure of thyroid-fed rats on a soybean-containing ration to develop a deficiency of the "antithyrototoxic factor of liver" may be due to the presence of such a factor. Recent findings (Rubin and Bird, '47) indicate that diets high in soybean meal increase body requirements for vitamin B<sub>12</sub>. The combination of such a diet and an increased vitamin B<sub>12</sub> requirement resulting from thyroid feeding (Bethel and Lardy, '49) would appear to be primarily responsible for the appearance of a vitamin B<sub>12</sub> deficiency in hyperthyroid rats on a soybean-containing ration.

The pretest dietary regime is also of importance in conditioning responses to thyroid feeding. Since significant amounts of vitamin B<sub>12</sub> can be stored in the tissues of the young rat during the period of pregnancy and lactation (Emerson et al., '49), the occurrence of a vitamin B<sub>12</sub> deficiency and its extent following thyroid feeding is dependent in part on the vitamin B<sub>12</sub> content of the pretest dietary regime. The growth retardation of hyperthyroid rats fed a diet low in fat and containing casein as the dietary protein was completely counteracted in the present experiments by extracted liver residue. Vitamin B<sub>12</sub> under similar experimental conditions showed no growth-promoting activity. It would appear, therefore, that the growth retardation noted above was due entirely to a factor or factors other than vitamin B<sub>12</sub>. Apparently, sufficient amounts of vitamin B<sub>12</sub> were stored by the animals from their

pretest dietary regime to meet any increase in vitamin B<sub>12</sub> requirements resulting from thyroid feeding. Bethel and Lardy ('49) recently reported that vitamin B<sub>12</sub> *partially* counteracted the growth retardation of male rats fed thyroid-containing rations similar to those described above. It would appear that these workers were dealing with a deficiency of both vitamin B<sub>12</sub> and the "antithyrototoxic factor of liver." Further work is needed to determine to what extent differences in sex, strain and pretest dietary regime were responsible for the diverse results obtained by Bethel and Lardy and by the present author.

#### SUMMARY

Full-fat soybean meal completely counteracted the growth retardation of immature rats fed massive doses of desiccated thyroid or iodinated casein. It did not counteract, however, the attendant reduction in basal metabolic rate.

The antithyrototoxic effects of full-fat soybean meal were correlated in part with its fat content. Growth was retarded in hyperthyroid rats fed similar diets but containing low fat soybean flour in place of the high fat soybean meal.

The retardation in growth of immature hyperthyroid rats on a diet containing low fat soybean flour was completely counteracted by the administration of either soybean oil, extracted liver residue or crystalline vitamin B<sub>12</sub>. When immature rats were fed a similar diet but containing casein as the dietary protein, the growth retardation following thyroid feeding was completely counteracted only by extracted liver residue. Soybean oil showed some counteracting activity; vitamin B<sub>12</sub> was inactive.

Increasing the fat content of the diet augmented significantly the gains in body weight of hyperthyroid rats fed a diet low in fat and containing casein as the dietary protein. Both animal and vegetable fats were active in this respect.

cantly more weight on a ration containing low fat soybean flour as the dietary protein than they did on a diet containing purified casein. Since the amount of thyroid in both diets was identical, it is apparent that the soybean flour either: (1) contained one or more factors which counteracted in part the growth-retarding effects of thyroid; or (2) stimulated the synthesis of such a factor or factors by the intestinal flora or the animal's own tissues.

Although part of the growth-promoting effect of low fat soybean flour may have been due to its fat content (7%), it would appear that it contains at least one additional factor which is qualitatively similar to, if not identical with, the "antithyrotoxic factor of liver." The failure of thyroid-fed rats on a soybean-containing ration to develop a deficiency of the "antithyrotoxic factor of liver" may be due to the presence of such a factor. Recent findings (Rubin and Bird, '47) indicate that diets high in soybean meal increase body requirements for vitamin B<sub>12</sub>. The combination of such a diet and an increased vitamin B<sub>12</sub> requirement resulting from thyroid feeding (Bethel and Lardy, '49) would appear to be primarily responsible for the appearance of a vitamin B<sub>12</sub> deficiency in hyperthyroid rats on a soybean-containing ration.

The pretest dietary regime is also of importance in conditioning responses to thyroid feeding. Since significant amounts of vitamin B<sub>12</sub> can be stored in the tissues of the young rat during the period of pregnancy and lactation (Emerson et al., '49), the occurrence of a vitamin B<sub>12</sub> deficiency and its extent following thyroid feeding is dependent in part on the vitamin B<sub>12</sub> content of the pretest dietary regime. The growth retardation of hyperthyroid rats fed a diet low in fat and containing casein as the dietary protein was completely counteracted in the present experiments by extracted liver residue. Vitamin B<sub>12</sub> under similar experimental conditions showed no growth-promoting activity. It would appear, therefore, that the growth retardation noted above was due entirely to a factor or factors other than vitamin B<sub>12</sub>. Apparently, sufficient amounts of vitamin B<sub>12</sub> were stored by the animals from their

pretest dietary regime to meet any increase in vitamin B<sub>12</sub> requirements resulting from thyroid feeding. Bethel and Lardy ('49) recently reported that vitamin B<sub>12</sub> *partially* counteracted the growth retardation of male rats fed thyroid-containing rations similar to those described above. It would appear that these workers were dealing with a deficiency of both vitamin B<sub>12</sub> and the "antithyrototoxic factor of liver." Further work is needed to determine to what extent differences in sex, strain and pretest dietary regime were responsible for the diverse results obtained by Bethel and Lardy and by the present author.

#### SUMMARY

Full-fat soybean meal completely counteracted the growth retardation of immature rats fed massive doses of desiccated thyroid or iodinated casein. It did not counteract, however, the attendant reduction in basal metabolic rate.

The antithyrototoxic effects of full-fat soybean meal were correlated in part with its fat content. Growth was retarded in hyperthyroid rats fed similar diets but containing low fat soybean flour in place of the high fat soybean meal.

The retardation in growth of immature hyperthyroid rats on a diet containing low fat soybean flour was completely counteracted by the administration of either soybean oil, extracted liver residue or crystalline vitamin B<sub>12</sub>. When immature rats were fed a similar diet but containing casein as the dietary protein, the growth retardation following thyroid feeding was completely counteracted only by extracted liver residue. Soybean oil showed some counteracting activity; vitamin B<sub>12</sub> was inactive.

Increasing the fat content of the diet augmented significantly the gains in body weight of hyperthyroid rats fed a diet low in fat and containing casein as the dietary protein. Both animal and vegetable fats were active in this respect.

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## MEAD JOHNSON AND COMPANY 'B-COMPLEX' AWARD

Nominations are solicited for the 1950 Award of \$1000.00 established by Mead Johnson and Company to promote researches dealing with the B-complex vitamins. The recipient of this Award will be chosen by a Committee of Judges of the American Institute of Nutrition and the formal presentation will be made at the annual meeting of the Institute in the spring of 1950.

The Award will be given to the laboratory or clinical research worker in the United States or Canada who, in the opinion of the judges, has published during the previous calendar year, January 1 to December 31, the most meritorious scientific report dealing with the field of the 'B-complex' vitamins. While the award will be given primarily for publication of specific papers, the judges are given considerable latitude in the exercise of their function. If in their judgment circumstances and justice so dictate, it may be recommended that the award be made to a worker for valuable contributions over an extended period but not necessarily representative of a given year. Membership in the American Institute of Nutrition is not a requisite of eligibility for the award.

To be considered by the Committee of Judges, nominations for this award for work published in 1949 must be in the hands of the Chairman of the Nominating Committee by January 15, 1950. The nominations should be accompanied by such data relative to the nominee and his research as will facilitate the task of the Committee of Judges in its consideration of the nomination.

W. H. SEBRELL, JR.  
*Experimental Biology and  
Medicine Institute  
National Institutes of Health  
Bethesda, Maryland*

CHAIRMAN, NOMINATING COMMITTEE

## BORDEN AWARD IN NUTRITION

Nominations are solicited for the 1950 Award of \$1000.00 and a gold medal made available by the Borden Company Foundation, Inc. The American Institute of Nutrition will make this award in recognition of distinctive research by investigators in the United States and Canada which has emphasized the nutritive significance of the components of milk or of dairy products. The award will be made primarily for the publication of specific papers, but the judges may recommend that it be given for important contributions over an extended period of time. The award may be divided between two or more investigators. Employees of the Borden Company are not eligible for this honor.

The formal presentation will be made at the annual meeting of the Institute in the spring of 1950. To be considered for the award, nominations must be in the hands of the Chairman of the Nominating Committee by January 15, 1950. The nominations should be accompanied by such data relative to the nominee and his research as will facilitate consideration for the award.

L. A. MAYNARD

*Cornell University, Ithaca, New York*

CHAIRMAN, NOMINATING COMMITTEE

## OSBORNE AND MENDEL AWARD

Nominations are invited for the Osborne and Mendel Award of \$1000.00 established by the Nutrition Foundation, Inc. for the recognition of outstanding accomplishments in the general field of exploratory research in the science of nutrition. It shall be given to the investigator who, in the opinion of a Jury of Award, has made the most significant published contribution in the year preceding the annual meeting of the Institute, or who has published a series of contemporary papers of outstanding significance.

The Award will be presented at the annual meeting of the American Institute of Nutrition.

The recipient will be chosen by a Jury of Award of the American Institute of Nutrition. As a general policy, the Award will be made to one person. If, in the judgment of the Jury of Award, an injustice would otherwise be done, it may be divided among two or more persons. Normally preference will be given to research workers in the United States and Canada, but investigators in other countries, especially those sojourning in the United States or Canada for a period of time, are not excluded from consideration. Membership in the Institute of Nutrition is not a requirement for eligibility and there is no limitation as to age.

Nominations may be made by anyone. Nominations for the 1950 Award, accompanied by data relative to the accomplishments of the nominee, must be sent to the Chairman of the Nominating Committee before January 15, 1950.

H. E. CARTER  
*University of Illinois*  
*Urbana, Illinois*

CHAIRMAN, NOMINATING COMMITTEE



# OBSERVATIONS ON FOLIC ACID DEFICIENCY IN THE CHICK IN THE PRESENCE OF VITAMIN B<sub>12</sub><sup>1</sup>

C. A. NICHOL, L. S. DIETRICH, C. A. ELVEHJEM AND E. B. HART  
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SEVEN FIGURES

(Received for publication June 3, 1949)

Previous work has shown that chicks placed on a purified diet deficient in folic acid failed to grow normally and developed some degree of anemia. The administration of vitamin B<sub>12</sub> or refined liver extract alone had no effect on hemoglobin regeneration (Nichol, Harper and Elvehjem, '49b) but did produce some growth response. When these chicks were kept on the folic acid deficient ration for several weeks following treatment with liver extract or vitamin B<sub>12</sub>, they developed body tremors, extended, quivering wings and finally complete paralysis. None of these symptoms appeared in the control groups. Since the number of chicks used in the experiments

<sup>1</sup>Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by funds supplied by the Commercial Solvents Corporation, Terre Haute, Ind.; by Swift and Co., Chicago, Ill.; and by the Research Committee of the Graduate School from funds supplied by the Wisconsin Alumni Research Foundation.

We are indebted to Merck and Co., Inc., Rahway, N. J., for crystalline vitamin B<sub>12</sub>, the vitamin B<sub>12</sub> concentrate and crystalline vitamins; to the Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y., for synthetic folic acid; to Abbott Laboratories, North Chicago, Ill., for haliver oil; to Wilson and Co., Inc., Chicago, Ill., for gelatin; to Allied Mills, Inc., Peoria, Ill., for soybean oil; and to E. I. du Pont de Nemours and Co., Inc., New Brunswick, N. J., for crystalline vitamin D.

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on hemoglobin regeneration was limited, the growth of folic acid deficient chicks in the presence of vitamin B<sub>12</sub> and the occurrence and cure of the observed symptoms were investigated further.

#### EXPERIMENTAL

Straight-run crossbred chicks (New Hampshire ♂♂ × Single Comb White Leghorn ♀♀), which were the progeny of hens fed diet B-1<sup>2</sup> described previously (Robblee et al., '48), were used in all experiments. The chicks were housed in electrically heated batteries with raised screen floors. Feed and water were supplied ad libitum. The chicks were wing-banded when one day old. Weights were recorded at weekly intervals.

The purified basal ration contained sucrose 61 gm, alcohol-extracted casein 18 gm, gelatin 10 gm, salts V 6 gm (Briggs et al., '43), soybean oil 5 gm, L-cystine 0.3 gm, thiamine hydrochloride 0.3 mg, riboflavin 0.6 mg, pyridoxine hydrochloride 0.4 mg, nicotinic acid 5.0 mg, calcium pantothenate 2.0 mg, biotin 0.02 mg, inositol 100 mg, choline chloride 150 mg, 2-methyl-1, 4-naphthoquinone 0.05 mg,  $\alpha$ -tocopherol 0.3 mg, and fortified haliver oil (60,000 U.S.P. units of vitamin A, 6,000 U.S.P. units of vitamin D<sub>3</sub> per gram) 40 mg.

Liver extract and vitamin B<sub>12</sub> were compared with respect to their effect upon growth and the incidence of the symptoms (table 1). All chicks were fed the folic acid deficient basal ration for 19 days. Then one group received one U.S.P. unit of liver extract<sup>3</sup> by intramuscular injection on alternate days. Another group was fed a vitamin B<sub>12</sub> concentrate mixed into the ration to provide approximately 3  $\mu$ g of vitamin B<sub>12</sub> per 100 gm of ration. A smaller group given no dietary supplement served as a negative control.

During a two-week period both liver extract and vitamin B<sub>12</sub> stimulated the growth rate of the folic acid deficient chicks.

<sup>2</sup>Ground yellow corn 36 lb., ground wheat 25 lb., ground oats 10 lb., wheat bran 5 lb., wheat middlings 5 lb., alfalfa leaf meal 5 lb., fish meal 2 lb., soybean oil meal 2 lb., meat scraps 2 lb., bone meal 1 lb., iodized salt 0.5 lb., fish oil (85D-600A) 1 lb., MnSO<sub>4</sub>·H<sub>2</sub>O 0.2 oz., riboflavin 100 mg.

<sup>3</sup>Lilly, Reticulogen, 20 U.S.P. units per milliliter.



Symptoms similar to those observed in previous experiments occurred in 54% of the chicks receiving liver extract and in 52% of those receiving vitamin B<sub>12</sub>.

As the symptoms developed, the wings were first extended from the sides of the body and quivered persistently in the position shown in figure 1. A sudden rigid extension of the toes occurred when the birds were picked up, and tremors of the whole body were noticed. The extension of the wings was periodic at first but became more pronounced and was accompanied by unsteadiness and difficulty in maintaining balance when walking (fig. 2). The birds consistently fell forward,

TABLE 1

*The effect of liver extract and vitamin B<sub>12</sub> concentrate  
on folic acid deficient chicks*

SUPPLEMENT	AVERAGE WEIGHT		NO. OF CHICKS	INCIDENCE OF SYMPTOMS
	19 days	33 days		
None	gm 70	gm 79	14	% 0
Liver extract (0.5 U.S.P. unit per bird per day, injected)	71	115	83	54
Vitamin B <sub>12</sub> concentrate, 0.7% (contributes 3 µg vitamin B <sub>12</sub> per 100 gm of ration)	70	109	50	52

and in severe cases they would lie prostrate, apparently paralyzed. However, when the leg muscles were exercised and the bird was lifted to its feet partial recovery occurred. The chick could then stand and walk for some time, although the extended quivering wings persisted. The birds remained in a paralyzed condition for two to 4 days before dying.

The incidence of extended wings and paralysis was higher among the more rapidly growing birds within the groups which were supplemented with vitamin B<sub>12</sub> alone. The chicks placed on test in order that the response of the symptoms to various treatments might be observed were generally 20 gm heavier than the average weight for the whole group.

The severity of the symptoms was graded on an arbitrary scale from zero (normal) to +++++ (prostrate), and on this basis appropriate groups of chicks were compared. The condition approximating grade +++ is shown in figure 2.

The response of the chicks to treatment with folic acid is shown in figure 3. Each curve represents the average grade of the number of chicks indicated inside the brackets. The control group showed a consistently severe condition; by the 13th

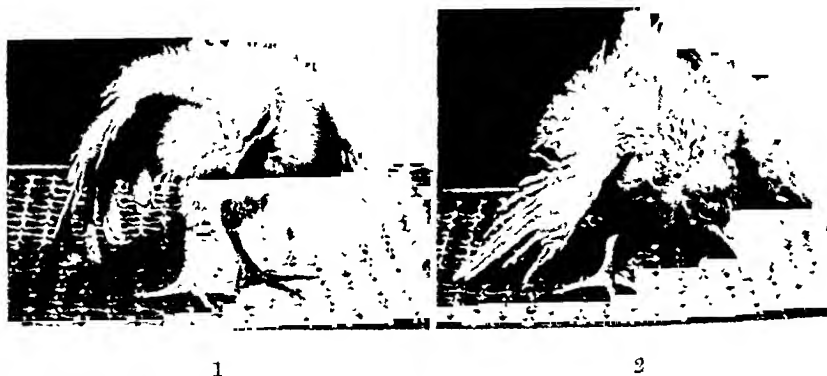


Fig. 1 (left) — Folic acid deficient chick receiving vitamin B<sub>12</sub> (age—5 weeks, weight 88 gm), showing the early periodic extension of the wings, which quiver when held in this position.

Fig. 2 (right) — Folic acid deficient chick receiving vitamin B<sub>12</sub> (age—5 weeks, weight 106 gm), showing the persistent extension of the wings and inability to maintain balance.

day, 4 of the 12 chicks in this group had died. All of the others survived the experimental period shown. Injection of 50  $\mu$ g of folic acid caused complete recovery for 12 to 14 days, at which time the symptoms reappeared and progressed rapidly to a severe condition. Injection of 20  $\mu$ g of folic acid caused marked improvement for 6 to 8 days. Fifty micrograms of folic acid administered orally were not as effective as 20  $\mu$ g given by injection. All doses were administered only once, on the first day (indicated by zero) of the experimental period shown in figure 3.

Other substances tested which had no curative effect when administered orally included: *p*-aminobenzoic acid (25 mg/bird/day), ascorbic acid (50 mg/bird/day), thymine (2 mg/bird/day), adenine and guanine (2 mg of each/bird/day) and 60% methanol extract of fresh liver (0.3 ml 3 gm fresh liver/bird/day). A concentrate of the *Leuconostoc citrovorum* factor<sup>4</sup> (Sauberlich and Baumann, '48), administered by injection, was also inactive (11,000 units/bird/day).

The growth-promoting activity of vitamin B<sub>12</sub> was tested in the presence and absence of folic acid (table 2). Also the activity of a vitamin B<sub>12</sub> concentrate mixed into the ration

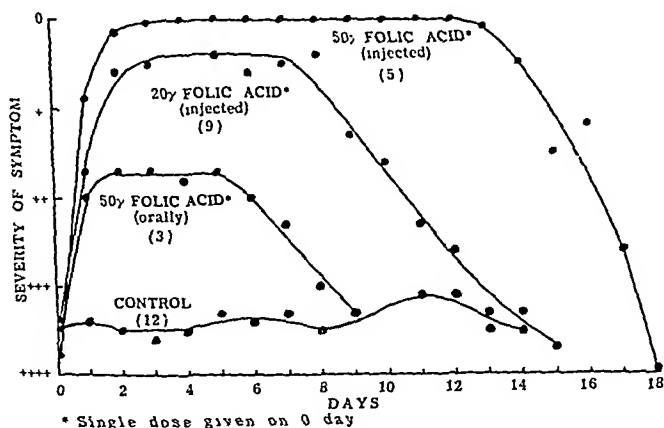


Fig. 3 Response of deficient chicks to treatment with folic acid.

was compared with that of pure vitamin B<sub>12</sub> administered by injection. In the groups which were supplemented during the 4-week period, vitamin B<sub>12</sub> caused a significant increase in the rate of growth when added to the folic acid deficient basal ration (groups 1 and 2) or when added to a ration containing 200 μg of folic acid per 100 gm (groups 3 and 4). Crystalline vitamin B<sub>12</sub> (0.1 μg/bird/day injected intramuscularly during the third and 4th weeks) stimulated the rate of growth (group 6) to the same extent as the vitamin B<sub>12</sub> concentrate which

<sup>4</sup>We are indebted to G. R. Drysdale and C. A. Baumann for a concentrate of the *Leuconostoc citrovorum* factor.



Fig. 4 No foliæ, no B<sub>12</sub> (av. wt., 98 gm.).



Fig. 5 No foliæ, plus B<sub>1</sub> (av. wt., 166 gm.).



was added to the folic acid deficient ration (group 5) during the same period.

The characteristic symptoms described above developed in all groups which received vitamin B<sub>12</sub> without folic acid, whether as a concentrate mixed into the ration or as the crystalline material administered by injection. These symptoms were observed in 66% of the chicks in group 2, 52% in group 5 and 55% in group 6. None of these symptoms

TABLE 2  
*The effect of vitamin B<sub>12</sub> when given with and without folic acid to chicks fed a purified ration*

GROUP NO.	SUPPLEMENT	NO. OF CHICKS	Hb 4 WEEKS <sup>1</sup>	AV. WT. 4 WEEKS
			gm %	gm
1	None	15	7.26	75
2	B <sub>12</sub> (concentrate, 0.7% $\equiv$ 3 $\mu$ g per 100 gm of ration)	17	6.74	128
3	Folic acid (200 $\mu$ g per 100 gm of ration)	19	8.09	195
4	Folic acid (200 $\mu$ g per 100 gm) + B <sub>12</sub> (3 $\mu$ g per 100 gm)	16	8.10	237
5	B <sub>12</sub> (concentrate, 0.7% $\equiv$ 3 $\mu$ g per 100 gm of ration) <sup>2</sup>	71	6.64	94
6	B <sub>12</sub> (crystalline, 0.1 $\mu$ g per bird per day, injected) <sup>2</sup>	11	6.47	105

<sup>1</sup>Ten observations per group.

<sup>2</sup>Vitamin B<sub>12</sub> given during the third and 4th weeks.

appeared in the negative control group (group 1) or in the groups which received folic acid (groups 3 and 4).

The effects of folic acid and of vitamin B<sub>12</sub> upon feathering are compared in figures 4-7, which show the typical feathering of chicks in groups 1 to 4, table 2, at the age of 5 weeks. The retarded growth rate and poor feathering of the chicks receiving only the basal ration is characteristic of folic acid deficiency (Mills et al., '42). The addition to the folic acid deficiency ration of vitamin B<sub>12</sub> alone increased

the rate of growth but did not cause comparable improvement in feathering. When folic acid ( $200\text{ }\mu\text{g}/100\text{ gm}$ ) was added to the basal ration, normal feathering was observed. Chicks which received both folic acid and vitamin  $B_{12}$  also showed normal feathering but grew more rapidly than the birds which were supplemented with folic acid alone.

#### DISCUSSION

Briggs et al. ('43) occasionally observed paralysis in chicks receiving the basal ration used in a study of vitamins  $B_{10}$  (for feathering) and  $B_{11}$  (for growth). They described the "trembling body and wings" and a temporary recovery from the paralyzed condition following exercise, which indicate the similarity to the symptoms described here. No effort was made at that time to secure chicks from a uniform source. The now-recognized variable growth response due to vitamin  $B_{12}$  stored by chicks from commercial sources was very likely a factor in the occurrence of the symptoms.

The early work on the separation of vitamins  $B_{10}$  and  $B_{11}$  was undoubtedly complicated because: (1) the occasional observation of the above symptoms indicated a lack of uniformity with respect to the vitamin  $B_{12}$  stored in the chicks; (2) varying amounts of vitamin  $B_{12}$  would occur in the fractions which were derived from solubilized liver; and (3) variation with respect to both growth and feathering would occur because of the marginal amounts of folic acid present in many of the fractions. The presence of two factors was clearly indicated by the data presented, but interpretation of the data was made difficult by inability completely to separate growth from feathering and by the different activities of the conjugated forms of folic acid in the microorganisms and in the chick.

Present knowledge of the activity of folic acid and vitamin  $B_{12}$  in the chick permits better interpretation of the earlier work on vitamins  $B_{10}$  and  $B_{11}$ . A purified ration supplemented with folic acid supports good growth and normal feathering. The addition of vitamin  $B_{12}$  and folic acid to the basal ration

also supports normal feathering, yet further increases the rate of growth. Chicks receiving the folic acid deficient basal ration showed the retarded growth and poor feathering earlier attributed to vitamin B<sub>10</sub> deficiency. The addition of vitamin B<sub>12</sub> to this basal ration increased the rate of growth but caused only slight improvement in feathering.

The occurrence of the extended wings and of paralysis was related to the growth rate induced by vitamin B<sub>12</sub> in the folic acid deficient chicks. Richardson et al. ('45) have described a similar condition in turkey poults receiving a vitamin B<sub>c</sub> (folic acid) deficient ration. The folic acid requirement of the more rapid-growing poult is reported to be at least twice that of the chick (Jukes et al., '47). The poults which showed spastic cervical paralysis and drooped, quivering wings had completely recovered one day following treatment with folic acid. Two observations of Richardson et al. ('45) bear important relationship to the work presented here: (1) more rapid development of cervical paralysis occurred when a liver extract containing only traces of folic acid was added to the cerelese-casein-gelatin basal ration; and (2) the symptoms developed several weeks sooner in poults hatched from hens without access to forage than in those from hens on range. The chicks used in the present studies were depleted by control of the hens' diet, and were the same as those which have shown consistent growth responses to treatment with liver extracts (Nichol et al., '49c) and crystalline vitamin B<sub>12</sub> (Nichol, Dietrich, Cravens and Elvehjem, '49a).

In all experiments in which chicks receiving the folic acid deficient basal ration were supplied with vitamin B<sub>12</sub> as the crystalline material, or with liver extract by injection or as a concentrate mixed into the ration, the characteristic symptoms described above developed in more than 50% of the birds. None of these symptoms appeared in the control groups. Treatment with adequate folic acid caused their rapid and complete disappearance.

The use of the crude charcoal concentrate of vitamin B<sub>12</sub> left open the possibility of contamination with folic acid. In

the experiments here reported this vitamin B<sub>12</sub> concentrate was compared with liver extract and crystalline vitamin B<sub>12</sub>. The similar growth response and the consistent development of symptoms precludes explanation of the observed growth by the presence of folic acid.

The livers of severely depleted chicks were found to contain approximately one-half the concentration of folic acid that is present in the livers of normal birds. Any action of vitamin B<sub>12</sub> resulting in more efficient utilization of folic acid within the tissues, or in stimulating the production of folic acid by intestinal microorganisms, should be reflected by changes in the growth rate and hemoglobin level. However, the increased growth was not accompanied by improvement in the anemia and poor feathering which are typical of folic acid deficiency in the chick. Also, folic acid caused disappearance of the symptoms characterized by the extended quivering wings before any change in hemoglobin level became apparent, and the high blood glucose observed in the chicks receiving the folic acid deficient basal ration was not lowered by the addition of vitamin B<sub>12</sub> alone to the diet (Williams et al., '49). The mechanism by which vitamin B<sub>12</sub> increases the growth rate may function independently of folic acid.

Although vitamin B<sub>12</sub> has been shown to have animal protein factor activity (Ott et al., '48), other compounds possessing similar activity may exist. Smith ('48) has reported the purification of two pigments from liver which have antipernicious anemia activity. Several unidentified, microbologically active components of liver extracts and fermentation products have been separated by means of paper chromatography (Cuthbertson and Smith, '49; Winsten and Eigen, '49). New and special conditions may be required to demonstrate the activity of these factors in the animal, but the possibility of their influence should not be overlooked.

#### SUMMARY

Folic acid deficiency in chicks fed a purified ration was accentuated by supplying vitamin B<sub>12</sub> as the crystalline ma-



terial, by injection or as a concentrate added to the ration. Extended quivering wings, body tremors and complete paralysis developed in more than 50% of the chicks which received vitamin B<sub>12</sub>. Treatment with folic acid caused complete disappearance of these symptoms within 24 hours. Injection of folic acid was more effective than oral administration. None of these symptoms developed in the control groups.

Vitamin B<sub>12</sub> increased the rate of growth of the folic acid deficient chicks but did not cause marked improvement in feathering. The addition of vitamin B<sub>12</sub> to a sucrose-casein purified ration containing 200 µg of folic acid per 100 gm caused a further significant increase in growth. The relation of folic acid and vitamin B<sub>12</sub> to the previously described vitamins B<sub>10</sub> and B<sub>11</sub> is briefly discussed.

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# THE PRODUCTION OF ANTIBODIES IN PROTEIN DEPLETED AND REPLETED RABBITS <sup>1</sup>

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ONE FIGURE

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## INTRODUCTION

The production of antibodies in the rabbit has been demonstrated over the years by various methods and with a wide variety of antigens. Little attention was paid to the diet in these studies, and the condition of the experimental animal was not considered in more than a general way. Of late years, however, our knowledge of nutrition has increased and its relation to immunity and resistance has been stressed (Cannon, '42). We have come to realize that the problem of antibody production is closely associated with the nutritional state of the animal.

Cannon, Chase and Wissler ('43) and Wissler ('47), using a low protein cookie plus carrots, have demonstrated that rabbits depleted in proteins on such a diet produced agglutinins of a much lower titer than rabbits on the usual commercial diet. These investigators studied the problem in depleted animals by means of agglutinin titers, which are end point determinations that test the relative concentration but not total antibodies of an antiserum.

For comparable quantitative studies, however, the full range of antigen antibody reactions must be considered (Boyden and

<sup>1</sup>These studies were supported by the Protein Metabolism Fund of the Bureau of Biological Research, Rutgers University, New Brunswick, N. J.

De Falco, '43; Boyden, Bolton and Gemeroy, '47), and unless the whole curve representing such reactions is taken into consideration, erroneous conclusions may be drawn with regard to the total antibodies produced. There is need to extend such studies to measure quantitatively the production of antibodies in rabbits at the end of an extended period of protein depletion, and again after repletion with a specific protein.

Since a simplified diet for the rabbit has not been formulated, it was necessary to try and develop a new diet that would maintain the animals through a lengthy depletion period as well as through the repletion period to follow.

#### METHODS AND MATERIALS

Since adult animals have been found to be better antibody producers than young ones (Freund, '30, '31) and are seemingly better able to withstand the rigors of long-term depletion, mature animals weighing around 3 kg and about three months old were used. These rabbits, of hybrid strains obtained from a local source, were conditioned for two weeks on a commercial diet prior to the start of the experiments.

Beef serum was used throughout the experiments as an antigen. The lyophilized beef serum<sup>2</sup> was dissolved in distilled water, Seitz-filtered, bottled under sterile conditions, labeled and stored in an icebox.

Many experiments at this laboratory<sup>3</sup> have shown that an intravenous injection of 1 ml of antigen given three to 4 weeks before the regular series is administered enhances the production of antibodies. Such a method we have called the presensitizing technique,<sup>4</sup> and the initial injection the presensitizing injection. The regular series consisted of 4 1-ml injections of the antigen, given subcutaneously every second day.

<sup>2</sup> Obtained through the courtesy of Sharp and Dohme, Inc., Research Laboratories, Glenolden, Pa.

<sup>3</sup> Unpublished data.

<sup>4</sup> Method suggested by Dr. R. J. DeFalco, Department of Zoology, Rutgers University, formerly with Sharp and Dohme, Inc., Glenolden, Pa.

One week after the last injection, blood was withdrawn from the ear artery in the required amounts. It was then stored in the icebox overnight, the sera being obtained by centrifugation next morning.

Tests for antibodies were carried out with these sera using the photoreflexometer, a photoelectric instrument which measures the turbidity developed in antigen antibody mixtures. In this method the antibody is kept constant and the antigen is diluted. The amount of precipitate formed is correlated with the readings on the galvanometer scale; thus the greater the antibody content of a serum the greater the precipitate and the higher the galvanometric reading (Libby, '38; Boyden and DeFalco, '43). Curves relating the antigen dilution with the galvanometric reading were plotted, the curve areas being a measure of the total turbidity of such reactions.

Total serum protein and non-protein nitrogen, hemoglobin and hematocrit determinations were carried out every three weeks throughout the experiment.

### *Preliminary experiments*

In their search for an adequate depletion diet for rabbits Cannon and his co-workers ('43) developed a low protein cookie, to be supplemented by carrots. One hundred grams of cookies were given to adult animals every other day, supplemented by 100 gm of carrots on the alternate day. Although the rabbits usually consumed their carrots, only about 60% of the cookies were eaten (Wissler, '47).

In our preliminary experiments, 100 gm of carrots were given daily and 100 gm of a modified Cannon cookie every other day to deplete our animals. This modified cookie consisted of the following ingredients: dextrose and dextrin, each 18.5%; cornstarch 40%; commercial hydrogenated cottonseed oil,<sup>5</sup> 3.5%; cellu flour 15%; Wesson salts ('32) 4.5%. One hundred grams of these cookies contained 0.054 gm of nitrogen.

<sup>5</sup> Crisco.

On this cookie-plus-carrot diet the rabbits lost weight rapidly. With the continuation of the diet the animals became extremely weak and death resulted in several cases. When depletion studies are carried out, it is important to distinguish between a diet with a single factor lacking and one where multiple deficiencies exist. Thus, in the series discussed above, the diet was deficient in both proteins and vitamins. To eliminate a possible vitamin deficiency, the following vitamins were added to every 100 gm of the diet: thiamine 400–500  $\mu$ g; pyridoxine HCl 500–600  $\mu$ g; calcium-*D*-pantothenate 4 mg; niacin 10–12 mg; *L*-inositol 36 mg; choline 100 mg; riboflavin 600–700  $\mu$ g; Navitol<sup>6</sup> 1,665 I.U. vitamin A, 333 I.U. vitamin D. Since many of the vitamin requirements of the rabbit have not yet been studied adequately, the amounts used were based on the known vitamin requirements of the dog, rat and guinea pig, modified according to what was thought might best meet the vitamin requirements of the rabbit.

The feeding procedures were changed slightly, and instead of every second day, 100 gm of carrots were fed daily. With these changes, 4 animals were carried successfully through a three-months' depletion period. Using the cookie type of diet and substituting 15% casein for an equivalent amount of carbohydrates, we were, however, unable to replete them with equal success. Therefore a search was made for an uncooked diet palatable to rabbits and on which they could be depleted and repleted successfully. The aim was to develop a completely purified diet, but this was not achieved since the carrot supplement still had to be used. Without the carrots even this diet was not acceptable to most of the experimental animals.<sup>7</sup>

To maintain the animals in a good state of health upon depletion and repletion, a new diet was introduced, patterned

<sup>6</sup> Squibb.

<sup>7</sup> However, in experiments conducted since, 4 rabbits were maintained over a 6-week period on the 24% casein diet to which 3% alfalfa meal was added, with no supplement of carrots.

after the one developed by Allison and Anderson ('45) in their depletion studies of the dog. After many trials with varying amounts of agar and cellu flour, the diet described in table 1 was devised; it was readily consumed by the rabbits. They ate on the average 75 gm of this diet per rabbit per day during the depletion period, along with an average of 135 gm of carrots. This diet was also fortified by the vitamins listed above. Water was given ad libitum.

### *Preparation of the diet*

The diet is prepared as follows: the agar is dissolved in boiling water and commercial hydrogenated cottonseed oil<sup>8</sup> is melted into the solution. The dry ingredients are weighed and thoroughly mixed in a separate pan and added to the agar-cottonseed oil mixture after it has cooled down to about 50°C. This facilitates mixing and the production of an even-textured diet.

The mixing is carried out with an electric stirrer at high speed until a homogeneous mixture is obtained. When cooled down sufficiently, and before solidifying, the water-soluble vitamins are added.<sup>9</sup> The resulting substance is then poured into a pan and stored in the icebox. A solid diet results. Small pieces in the amounts required for feeding purposes can be readily cut. Navitol, one drop per 100 gm of diet, is added to each ration at the time of feeding

### *Caloric intake during protein depletion*

Careful records were kept of the daily amounts of food consumed by the rabbits. The caloric value of the low protein diet is given in table 1; the caloric value of 100 gm of carrots was estimated at 40 Cal.

The average caloric intake during the depletion period, as calculated per kilogram of body weight per day, is presented

<sup>8</sup> See footnote 5, page 301.

<sup>9</sup> In our previous diets the water-soluble vitamins were added, with the Navitol, on top of the ration. By adding these vitamins directly to the diet during its preparation, a better distribution is assured.

in table 2. This table also shows the weight losses suffered during this period, the average length of which was 98 days. During this time the rabbits consumed, on the average, 75 gm of diet and 135 gm of carrots per animal per day.

TABLE 1  
*Low protein diet*<sup>1</sup>

INGREDIENTS		DIET COMPOSITION
	gm %	gm
Dextrose	19.50	390
Dextrin	19.50	390
Cornstarch	42.16	843
Crisco	3.69	74
Cellu flour	7.38	148
Wesson salts	4.61	92
Agar	3.16	63
Total of dry ingredients	100.00	2,000
Water-plus-vitamin solution		2,800
Total		4,800

<sup>1</sup> One hundred grams of this diet contained 70 Cal. and 0.054 gm of nitrogen by micro Kjeldahl analysis.

TABLE 2  
*Average caloric intake per kilogram body weight and weights during the depletion and repletion periods*

RABBIT NO.	DEPLETION		REPLETION	
	Ave. Cal. intake per kg body weight	Weight		Ave. Cal. intake per kg body weight
		Start	End	
		gm	gm	gm
41	68.1	3,540	3,050	3,400
42	44.7	3,325	2,300	2,700
44	60.0	3,410	2,260	2,870
45	60.0	3,310	2,550	2,960
47	62.5	3,140	2,470	2,910
60	53.0	3,710	3,340	3,280
61	68.2	3,210	2,210	2,140
63	66.5	3,090	2,630	2,960
65	70.9	3,540	2,630	2,910
Ave. 61.5				Ave. 82.9



*Repletion diet and caloric intake during repletion*

Once this type of purified diet with an agar base was developed, it was easy to convert it into a repletion diet simply by substituting 24% casein for an equivalent amount of carbohydrates. The composition and caloric value of the protein repletion diet are given in table 3. This diet was enriched by vitamins in the same way as the depletion diet, and was also supplemented by carrots. The animals ate it readily, and in

TABLE 3  
*High protein diet*<sup>1</sup>

INGREDIENTS	DIET COMPOSITION	
	gm %	gm
Dextrose	13.61	272
Dextrin	13.61	272
Cornstarch	30.21	604
Crisco	3.53	71
Casein <sup>2</sup>	25.15	503
Cellu flour	6.82	136
Wesson salts	4.13	83
Agar	2.94	59
Total of dry ingredients	100.00	2,000
Water-plus-vitamin solution		2,800
Total		4,800

<sup>1</sup> The caloric value of this diet was 70 Cal. per 100 gm.

<sup>2</sup> Borden's vitamin-free Labco casein was used throughout the experiment.  
Average protein content: 82%.

experiments since conducted at our laboratories we have had little difficulty in feeding this 24% casein diet to rabbits even after they have been for many months on a commercial stock diet.

The average length of the repletion period was 37 days. During this time the rabbits consumed, on the average, 150 gm of diet and 100 gm of carrots per rabbit per day. The average caloric intake per kilogram of body weight per day was 82.9 Cal., as compared with 61.5 Cal. during the depletion period (table 4).

Three of the rabbits were kept for an additional period of 47 days on the 24% casein diet plus carrots. Their food and caloric intake ran close to the data reported above. During this additional 47-day period the animals consumed, on the

TABLE 4  
*The effects of diets on antibody response curve areas*

RABBIT NO.	AT THE END OF DEPLETION	AT THE END OF REPLETION
<i>Animals on depletion and repletion</i>		
	GROUP I	
41	1,099.5	2,363.0
42	114.5	726.5
44	87.0	360.5
45	131.5	466.0
47	498.0	1,859.0
	GROUP II	
60	389.5	1,464.5
61	186.0	872.0
63	891.0	2,530.0
65	824.5	2,398.5
	Ave. 469.1	Ave. 1,448.9
Increase due to high protein diet		
<i>Animals on commercial diet</i>		
70	296.5	276.0
71	1,286.5	1,890.0
72	184.5	308.0
73	458.5	649.0
74	244.0	84.5
	Ave. 494.0	Ave. 641.5
Increase due to multiple injection series		30.0%

average, 150 gm of diet and 115 gm of carrots per rabbit per day and the average caloric intake was 89.4 Cal. The animals gained weight steadily during this period and seemed to be in a good nutritional state when the experiment was discontinued.

These data seem to point to the 70 to 80 Cal. requirement of the rabbit per kilogram of body weight per day, which is in close agreement with the requirements already established for the dog and the rat.

## RESULTS

### *Effects of protein depletion and repletion upon serum protein and hematological values*

These values were measured every three weeks during the experimental period. As a general rule the concentration of serum proteins decreased as depletion progressed, though this was not as marked in some animals as in others. Hematocrit and hemoglobin values, however, had fallen to a greater degree in all the animals by the end of the depletion period. After 5 weeks' repletion the serum protein levels were higher than at the start of the experiment; hematocrit values were also higher in most cases, but hemoglobin values were still below those originally recorded in all but two cases. A 24% casein diet does not seem to be adequate for hemoglobin regeneration in rabbits over such a short repletion period.

### *Antibody response in depleted and repleted rabbits*

Figure 1 and table 4 show the effects of protein depletion and repletion on antibody production when beef serum was used as an antigen. One surprising result was observed with the animals depleted in proteins for a period of 98 days; even after this long depletion period some of the rabbits still produced very good antibodies (fig. 1-A). In fact, the curve areas representing the turbidities of the antigen antibody reactions are greater in some cases than those for the animals fed the usual commercial diet. When the depleted animals were repleted with the 24% casein diet plus carrots for a period of 5 weeks, the antibody response markedly increased (fig. 1-B). The differences in the curve areas obtained under similar experimental conditions show the wide variation of individual response that may be found in rabbits.

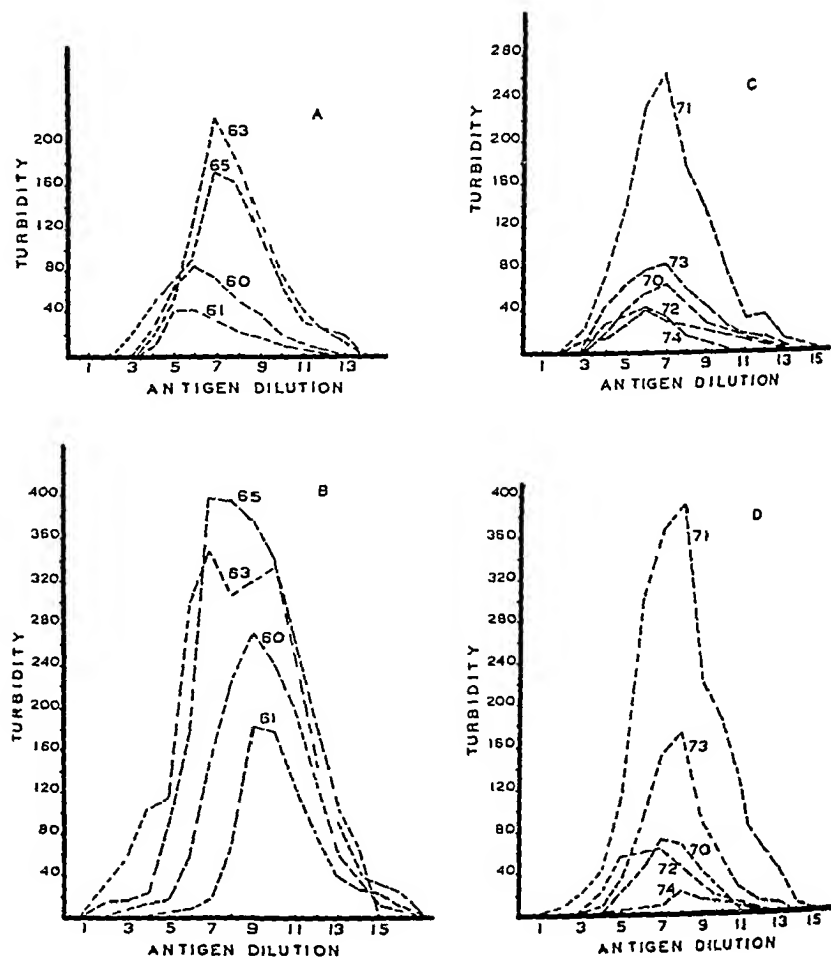


Fig. 1-A Antigen-antibody precipitation curves obtained with 4 protein depleted rabbits with vitamin supplements added to the diet (group II).

Fig. 1-B Antigen-antibody precipitation curves obtained with 4 rabbits repleted on a 24% casein diet for 37 days after a 97-day period of protein depletion (group II).

Fig. 1-C Antigen-antibody precipitation curves obtained with 5 rabbits fed a commercial diet, after a single injection series with beef serum.

Fig. 1-D Antigen-antibody precipitation curves obtained with 5 rabbits fed a commercial diet following a second series of injections with beef serum.

*Note:* Antigen dilution values represent the amount of protein in the antigen. The original dilution, marked "1" on the dilution scale in all figures, is one part of protein to 62.5 parts of saline. Turbidity is a measure of the antigen antibody reactions in terms of the galvanometric scale of the instrument used. The numbers on the curves identify individual rabbits used in the experiments.

It is generally agreed that a double injection series produces more antibodies than a single series; although this is not always the case, as is shown with respect to two of the rabbits fed a commercial diet. To see to what extent a double series of injections was responsible for the increase in antibodies, 5 rabbits fed a commercial diet were injected in the same manner and over the same period of time as the experimental animals. The increase due to the multiple injections series was 30.0% (fig. 1-C, 1-D), whereas the increase due to the high protein diet was 209.0% (table 4). Thus the diet was primarily responsible for the enhanced antibody production.

As was previously stated, all diet factors must be taken into consideration in depletion and repletion studies where antibody production is being measured. In our preliminary experiments the diet was deficient in vitamins as well as in proteins, and with the group of rabbits fed this diet our poorest antibody response was obtained. With the animals in groups I and II (table 4) whose diet was fortified with what might be considered the essential vitamins, the antibody response was much greater, indicating the important role of vitamins in antibody production. Stoerk and Eisen ('46) and Axelrod et al. ('47) have shown that a deficiency of pyridoxine and other vitamins in the diet markedly decreases the amount of circulating antibodies.

The value for antibody production of an adequate protein intake, which is here demonstrated, has been stressed before (Cannon et al., '43; Clausen, '34). It would seem, however, that if all other dietary constituents except protein are adequate, the rabbit is still able to produce good antibodies, as is shown in figure 1-A.

These results seem to indicate that a high-protein diet fortified with other essential constituents may be of great practical importance in the production of hyperimmune sera. Further studies on the relation of diet to antibody production are under way.

## SUMMARY

1. Antibody response in protein depleted and repleted rabbits to beef serum as antigen was quantitatively studied by means of the precipitin reaction.

2. Turbidity measurements of the precipitins formed over the whole reaction ranged were carried out on the Libby photonreflectometer.

3. The injection technique used for antibody production consisted of a presensitizing dosage of 1 ml of antigen given intravenously and followed by a regular series of 4 subcutaneous injections of the same amount of antigen on alternate days, three to 4 weeks later.

4. A new type of semi-purified diet in an agar base was developed that would carry rabbits through the depletion and repletion periods. This diet was supplemented by carrots throughout the experiments.

5. The average caloric intake of the rabbit was determined during the course of the experiments.

6. Antibody response is higher in repleted than in depleted animals. It is only slightly higher in rabbits fed a commercial diet than in the protein depleted animals.

7. A high protein diet supplemented by other essential dietary factors is valuable for the production of hyperimmune sera in rabbits.

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## EROSION OF MOLAR TEETH BY ACID BEVERAGES<sup>1,2</sup>

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The erosion of the teeth by acids contained in such food-stuffs as lemon juice has long been recognized (Pickerill, '12). McClelland found he could decalcify teeth if they were left in a solution at a pH of 4.5 for 12 hours (McClelland, '26). In regard to more acid media he stated, "Certainly the presence of a reaction of 3.5 and below, even if existing for only a few minutes, is a potential source of damage to the teeth."

The great increase in the consumption of cola beverages having a pH of 2.6 during the past 30 years has renewed interest in the effect of acids upon teeth. McClure ('43) in the case of rats and Stafne and Lovestedt in the case of man ('47) have called attention to the erosion of both the enamel and dentine by such acid beverages.

Wynn and Haldi ('48) have produced erosion of the enamel of the teeth of rats fed fruit juices that had only about one-tenth the hydrogen ion concentration of cola beverages. Their animals were fed the juices for long periods of 100 to 200 days. If the amounts they fed were related to the body weight of a man, their studies were equivalent to having a man ingest one-half to one ton of fruit juice during a period of 200 days.

<sup>1</sup>These studies were made under a grant from the Office of Naval Research, Contract N6ori-91, Task Order 6 (NR 182-077). Special assistance was given by Comdr. C. A. Schlack (D.C.) and his staff at N.M.R.I.

<sup>2</sup>Substantial technical assistance was rendered by Mrs. Audrey M. Ackerman and Janet S. Ricker.

In an earlier report (Gortner, McCay, Restarski and Schlack, '46) the effect of oxalate in preventing the acid erosion of teeth was described. Many other factors, such as the buffering capacity of the saliva, may serve to help prevent this erosion. Smith ('46) has found that even emotional conditions may play a part in the control of acid erosion by influencing the volume and composition of saliva.

The present study covers a series of experiments on acid erosion and its prevention. Rats were used, and the same techniques described earlier (Gortner et al., '46) of autoclaving and scoring teeth (Restarski, Gortner and McCay, '45), were employed.<sup>3</sup> A question has been raised concerning the suitability of the rat for the study of tooth erosion, since this animal may drink in a manner different from that of man. However, some earlier studies were made with monkeys and dogs (Bieri, McCay, Restarski and Gortner, '46) and erosion was similar in these species to that found in the rat. Monkeys are known to drink like man and have been taught to drink from a cup.

## RESULTS AND DISCUSSION

### *Minimum detectable erosion*

The degree of acid erosion is the result of several factors; namely, the hydrogen ion concentration of the solution, the titratable acidity, the buffer action within the mouth, and the type of anion.

Since the national consumption of soft drinks is now claimed to have a retail value in excess of \$700,000,000, a series of such drinks were analyzed at the Naval Medical Research Institute, but the analyses were not published. The cola beverages were found to be among the most acid and to contain 10% sucrose and 0.055% phosphoric acid with a pH of 2.6. In our earlier comparative tests it was found that the degree of erosion of the molars was identical in experimental

<sup>3</sup> The stock diet used in all these studies was the open formula dog feed made by the Grange League Federation, Canandaigua, New York.

animals given cola beverages from dispensing machines and in those given a solution of phosphoric acid (0.055%) and sucrose (10%) made with distilled water. In order to determine the minimum amount of this acid solution that would erode the teeth of rats, varying amounts were fed and the animals were killed at intervals starting after the first day. Control rats were given distilled water instead of the acid beverage.

Detectable erosion was produced when a rat had consumed 10 ml of the acid during 24 hours, or 5 ml daily for two days. A few organic acids in a 10% sucrose solution were similarly tested. Erosion could be detected after three daily feedings of 3 ml of 0.23% tartaric acid. Similarly, the effects of a single daily ingestion of two, three or 5 ml of 0.58% citric acid could be observed. Rats often refused to drink 4% solutions of acetic acid, even if the solution contained 20% sucrose. In a few cases, however, erosion was found after the ingestion of 35 ml of such solutions.

Brown deposits on both sides of all molars were observed in 5 rats of the 17 that were used in the acetic acid studies. These brown deposits have been observed in the course of various experiments. It is believed that they are due to something in the mineral mixtures fed the animals, but their exact cause is still unknown.

#### *The effects of long-continued ingestion of acid beverages*

In order to compare the effects of long-continued ingestion of phosphoric acid, in the concentration used in cola beverages, with those of natural juices, 4 groups of rats were selected at an age of 50 to 60 days. Each rat was fed a daily allowance of 10 ml of the substance being tested. One group of rats received orange juice, one tomato juice, one phosphoric acid-sucrose in the concentrations used in cola beverages, and one distilled water. Rats were killed at the end of 6 weeks, 4 months and 6 months.

At the end of 6 weeks no erosion could be detected in the teeth of the animals given distilled water or tomato juice. Some erosion was found in the rats fed orange juice, and the most extensive erosion was found in those fed phosphoric acid. At the end of 4 months the erosion was more marked in those given orange juice or phosphoric acid, with some etching of the molars in those fed tomato juice. After 6 months the erosion produced by the phosphoric acid solution was so severe that it could not be graded in the usual manner. The tops of the molars were only slightly above the gum line.

In all cases erosion by tomato juice was the least, with phosphoric acid exerting the greatest effect upon the teeth.

#### *Rate of erosion of teeth in phosphoric acid solutions*

In order to have an approximate value for the rate of erosion of human teeth in phosphoric acid — sugar solutions similar in composition to cola beverages, two series of trials were made by immersing extracted human teeth in (a) solutions containing 0.055% phosphoric acid and 10% sucrose, and (b) a cola beverage purchased from a dispensing machine in the local market. The roots of the teeth were first coated up to the gum line with paraffin.

The teeth were then placed in flasks containing 50 ml of either phosphoric acid solution or cola beverage. The solutions were analyzed for calcium after definite time intervals. The flasks were not shaken.

The data are summarized in table 1. Although they might seem to indicate a greater rate of erosion in the cola beverage, this is probably not significant due to variability among extracted teeth and the difficulty of selecting matched specimens.

#### *The buffer capacity of the mouth*

Numerous studies, such as those of Pickerill ('12), have been made of the titratable acidity of saliva after stimulation by various foods. Also, in recent years many reports have been made of the pH of saliva under various conditions.

Little attention, however, has been paid to the hydrogen ion concentration of the mouth after stimulation by acid beverages. Since two important factors in the erosion of teeth are pH and titratable acidity, it seemed worthwhile to measure the buffer action of the mouth.

A simple test was devised for this purpose. A solution similar to cola beverages in its content of acid and sugar was prepared with 10% sucrose and 0.055%  $\text{H}_3\text{PO}_4$ . Varying amounts of this solution were tested and 10 ml were found the most satisfactory to hold and agitate in the mouth for a half

TABLE 1

*Rate of solution of calcium from human teeth immersed in phosphoric acid or cola beverage*

TIME IN SOLUTION	MEAN WT. TOOTH		DISSOLVED	
	0.055% $\text{H}_3\text{PO}_4$	Cola	0.055% $\text{H}_3\text{PO}_4$	Cola
<i>hours</i>	<i>gm</i>	<i>gm</i>	<i>mg</i>	<i>Ca/gm tooth</i>
3	1.20	1.35	1.3	1.4
6	1.47	1.24	1.9	2.5
9	1.01	1.23	2.7	4.4
12	0.88	0.98	6.0	4.2
24	1.34	0.68	3.9	6.3
72	1.18	0.60	5.3	11.7
120	1.42	1.42	5.5	5.6
188	1.57	0.91	6.0	9.0
336	1.55	0.67	6.7	14.6

minute. After this mixture of solution and saliva was returned to a beaker, the pH was determined by using the glass electrode and Bogen's Universal Indicator. Both methods proved satisfactory.

This test has been made on several hundred individuals and repeated frequently, under varying conditions, on a selected group. The range of pH values found varies from 2.8 to 5.6. Among a group of 32 young naval officers, composed half of women and half of men, the mean value for the men was 4.2 and for the women 3.7. Among a group of 20 graduate students composed of 8 women and 12 men the mean values

were 3.8 for the women and 4.4 for the men. Many tests have indicated this modest difference between the sexes. It is not related to height or body weight.

This buffer power of the mouth in the presence of acid beverages remains quite constant throughout the day. The results of studies made in relation to time of eating are summarized in table 2.

Teeth are usually considered subject to erosion if the pH of the mouth is lower than 3.5. Since the mouth in some cases cannot buffer an acid beverage to this extent even after a half minute and with a modest amount of acid, it would seem possible that some individuals have conditions in their mouths which would lead readily to acid erosion.

Our laboratory has made no study of the teeth of heavy consumers of acid beverages. However, in the course of testing the buffering capacity of the mouth it has proved very difficult to continue studies upon the same individuals if testing is on consecutive days. After a few days the subjects develop such sensitive teeth that they have difficulty in chewing food, and hence dislike to continue as members of the group being studied.

### *The oxalate film*

Since Restarski (Gortner et al., '46) first observed an oxalate film on the teeth of rats whose diet contained small amounts of sodium oxalate, many questions have been raised concerning the formation and permanency of this coating. Seven experiments were run, employing 204 young rats, to determine the effect of feeding various levels of sodium oxalate, mixed with the diet or dissolved in an acid beverage.

When rats were fed a finely ground meal containing 0.2 or 0.3% sodium oxalate as a fine powder for 14 days and then allowed to drink 20 ml of cola beverage, there was partial protection inasmuch as islands of enamel remained without the usual etching down to the gum line. However, erosion was marked around these coated areas where the deposit had formed.

In order to study the deposition of the film, a meal diet was mixed to contain 10 mg of sodium oxalate per 10 gm of dry feed. This allowance was fed daily to each rat in a group for a period of 13 days. Individuals were killed starting after the first day, which allowed the ingestion of at least 10 mg of sodium oxalate.

In a few cases thin patches were observed after this first day. After two days and the ingestion of 20 mg of oxalate,

TABLE 2

*The buffer capacity of the mouth before and after eating  
(pH after holding acid beverage in mouth for 30 sec.)*

SUBJECT	P	M	D	G	L	M	S	
Women graduate students								
1/2 hr. before breakfast	3.9	2.9	3.0	4.4	3.4	3.3	3.0	
1/2 hr. after breakfast	3.7	2.9	3.1	5.3	4.5	3.5	5.9	
1/2 hr. before lunch	3.9	3.0	3.1	4.9	4.3	4.7	3.6	
1/2 hr. after lunch	3.7	3.1	3.6	4.8	3.4	4.4	3.1	
1/2 hr. before supper	3.2	2.9	3.8	4.8	5.1	3.4	3.1	
1/2 hr. after supper	3.5	2.8	3.7	5.3	3.7	3.7	3.4	
Before retiring	4.0	2.9	3.5	3.9	lost	4.8	3.3	
	L	T	K	Th	M	E	Lo	H
Men graduate students								
1/2 hr. before breakfast	4.9	3.0	4.8	3.2	5.3	3.5	3.9	4.7
1/2 hr. after breakfast	5.0	3.1	5.4	3.2	5.3	4.9	3.6	5.0
1/2 hr. before lunch	4.6	3.5	5.2	3.2	5.7	4.4	4.4	4.6
1/2 hr. after lunch	4.6	3.6	4.8	3.5	5.6	4.6	4.5	5.5
1/2 hr. before supper	4.9	3.4	4.4	3.5	5.8	4.7	4.5	5.2
1/2 hr. after supper	4.9	3.9	5.4	3.2	5.8	4.9	4.6	5.1
Before retiring	4.8	3.8	5.6	3.1	5.6	4.0	3.8	4.6

all rats showed deposits. Large patches of deposit were found on the teeth of the animals that had consumed 130 mg of oxalate during a period of 13 days. These deposits were usually much more marked on the upper molars than on the lower ones.

Various trials were made to determine if sodium oxalate dissolved in drinking water would produce a film that might afford protection at a later date against acid beverages. Fif-

teen milligrams of sodium oxalate consumed daily for 25 days afforded no protection when rats were subsequently given 15 ml of cola beverage daily for 4 to 7 days. Even the feeding of 10 ml of 0.1% sodium oxalate dissolved in a 10% sucrose solution in the morning, followed by a cola beverage solution 5 hours later in the afternoon, afforded little protection against acid erosion.

No method was discovered of using oxalate to prevent acid erosion which proved superior to that described in an earlier report (Gortner et al., '46). Earlier experiments were repeated by scoring the teeth of rats given 10 ml of a cola beverage daily for 28 days. Nine rats received this beverage in this amount daily, while another comparable 9 were given the beverage containing 0.1% of sodium oxalate. The average total erosion score was 16 for the rats receiving oxalate compared with 46 for the animals given the acid beverage without oxalate; these scores indicate the degree of erosion.

### *Rhubarb and lemon juice*

Since rhubarb juice has long been used as a beverage, and since it affords a natural source of oxalate that will counteract the acid erosion of lemon juice when the two are combined, trials were run to determine the useful combinations. Rhubarb juice was extracted and canned after the stalks were steamed in a pressure cooker. The leaves of the rhubarb were not included, although they are known to be rich in oxalic acid. All solutions tested were sweetened by the presence of 20% sucrose.

The test consisted of allowing individual rats 15 ml of the combined juices daily for 14 days. After this the rats were killed and the teeth scored in the usual manner. The ratios tested were rhubarb:lemon, 1:1, 1:4, and 1:9.

The results of these tests indicated that the ratio 1:1 afforded almost complete protection at the base of the teeth but allowed some erosion at the tips. The other ratios afforded no protection. No studies were made to determine whether



this protective effect of rhubarb juice was due solely to its content of oxalate.

If these combinations of juices were diluted as would be done for human consumption, the ratio of the rhubarb juice had to be increased.

### *Phytate and stearates as protective agents*

Since both phytic and stearic acids form insoluble calcium compounds, it seemed possible that these might provide protective films on teeth. Calcium phytate, to make a solution of 0.03%, was dissolved in a phosphoric acid-sucrose solution similar to a cola beverage. Two groups of rats containing 5 animals each were fed this solution in the amount of 20 ml per day for 8 and 14 days, respectively. At the end of these periods the rats were killed and the erosion of their teeth compared with that of animals fed the solution without the phytate. No evidence was found of protective action by the phytate.

Calcium phytate was also fed mixed with a meal so that each rat consumed 20 mg daily for 14 days. These rats were allowed to drink 20 ml of cola beverage on the 15th day. Controls not given the phytate but drinking the same amount of acid beverage were used. No evidence was found of film formation or protection by phytate under these conditions.

Stearic acid was tested using 8 groups of 5 rats each. The acid was mixed into the meal diet for 6 of these groups at the following levels: 50, 100, 200, 400, 800, and 1,600 mg%. Two groups were used for purposes of comparison; one was fed the basal diet alone and the other the basal diet mixed with 50 mg% of sodium oxalate.

Each rat was allowed 20 ml daily for two weeks of the usual phosphoric acid-sucrose solution. Examination of the teeth of these animals at the end of this period revealed the usual deposit from the sodium oxalate but no film nor protection in any rat fed the different levels of stearic acid.

These exploratory studies afford no evidence that either phytic or stearic acid will develop protective films against acid erosion.

### *The feeding of sodium oxalate to beef steers*

In the hope of getting enough of the deposit that forms on the teeth of animals fed sodium oxalate for analysis, the powdered oxalate was fed to 4 beef steers being fattened for slaughter. Each was fed 7 gm daily of powdered sodium oxalate mixed with the concentrate. This was continued for one month. After this period the steers were slaughtered and the jaws removed.

No evidence of any deposit was found, indicating either that conditions in the mouth prevented deposition or that some action such as rumination removed the deposit.

### *Toxicity of sodium oxalate to dogs*

Although the early literature (Kobert and Kussner, 1879) gave substantial attention to the toxicity of oxalic acid, modern research has devoted scant consideration to the subject. In the studies of Hammersten ('37), dietary oxalate seemed to play a minor role in the production of oxalate stones in rats. Other dietary factors such as magnesium and the fat soluble vitamins appeared to be more important.

In order to determine whether injury could be detected after feeding oxalate to animals daily for a substantial period, a study covering 15 months was begun. Five young, adult mongrel dogs weighing 26 to 36 lb. were fed 100 mg of powdered oxalate daily, mixed with the stock diet. Post mortem examination yielded no evidence of stone formation or pathological changes. These dogs were maintained on the same complete stock diet which was fed to the rats in the erosion studies.

### SUMMARY

In the case of the rat, erosion of the molar teeth can be detected after the consumption of 10 ml of acid solutions of the

same strength used in cola beverages. A comparative test of acid erosion during a period of 6 months indicated that tomato juice had the least effect and 0.055% phosphoric acid the most. Rat teeth subjected to phosphoric acid for this period were eroded nearly to the gum line and erosion was too severe for scoring in the conventional manner.

Human teeth suspended in cola beverage or its equivalent in sucrose-phosphoric acid gradually lose calcium in the course of two weeks.

The buffer capacity of the human mouth against cola beverages differs widely but is fairly consistent for a given individual. Even after a half minute's exposure some mouths cannot buffer the solution to a pH higher than 3.5, which is considered marginal for erosion.

A series of tests indicates that sodium oxalate ingested with food deposits patches on the teeth but is not as effective in the prevention of erosion as oxalic acid dissolved in an acid beverage. Rhubarb juice combined with equal parts of lemon juice protects the teeth against erosion.

Neither stearic nor phytic acids prevented erosion by acid beverages.

Oxalate patches could not be deposited on the teeth of steers during a period of one month's feeding of sodium oxalate.

No evidence of injury or stone formation was found after feeding adult dogs 100 mg of sodium oxalate daily for 15 months.

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# THE EFFECT OF SUPPLEMENTAL METHIONINE ON THE NUTRITIVE VALUE OF DIETS CON- TAINING CONCENTRATES OF THE SOYBEAN TRYPSIN INHIBITOR<sup>1</sup>

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The marked improvement which proper heat treatment exerts on the nutritive value of soybean protein has been observed by numerous investigators, including Osborne and Mendel ('17); Hayward, Steenbock and Bohstedt ('36); Wilgus, Norris and Heuser ('36); Fritz, Kramke and Reed ('47); Klose, Hill and Fevold ('48); Borchers, Ackerson and Mussehl ('48a), and others. It has been further established that the supplementation of raw soybean meal with methionine is an effective means of largely eliminating the marked discrepancy in nutritive value between raw and properly heated soybean meal (Hayward and Hafner, '41; Evans and McGin-

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nis, '46; Gerry, Carrick and Hauge, '48). The effectiveness of supplemental methionine in this respect has been generally attributed to the increased availability of the sulfur-containing amino acids in the heated soybean protein. Johnson, Parsons and Steenbock ('39) demonstrated in the rat that this difference in the availability of the sulfur-containing amino acids between raw and heat-processed soybean meals could not be explained by any difference in the amount of sulfur absorbed from the intestine. Based on their own observation that the effect of heat on soybean protein was to increase the rate of release of methionine to a greater extent than that of leucine and lysine, Melnick, Oser and Weiss ('46) have suggested that, in the case of raw soybean, methionine is released too slowly in the intestinal tract to be available for simultaneous utilization with the other essential amino acids.

Attention has recently been directed to the presence of one or more antitryptic factors in unheated soybean meal (Bowman, '44, '46, '48; Ham and Sandstedt, '44; Kunitz, '46, '47) and to the deleterious effect of trypsin inhibitor preparations on the growth of experimental animals (Ham, Sandstedt and Mussehl, '45; Klose, Hill, and Fevold, '46a; Borchers, Ackerson, and Mussehl, '48b). Westfall and Hauge ('48) were able to correlate the improvement in the nutritive value of soybean protein effected by heat treatment with destruction of trypsin inhibitor potency. Although these lines of investigation suggest that the poor growth-promoting value of raw soybean meal is due to an inhibition of proteolytic digestion in the intestine, affecting the enzymatic release of methionine, recently reported experiments by Desikachar and De ('47), Klose, Greaves and Fevold ('48), and Westfall, Bosshardt and Barnes ('48) have demonstrated the growth-depressing effect of active antitryptic preparations even when incorporated into diets containing pre-digested protein.

The present investigation was undertaken in an attempt to clarify the mechanism of this anomalous behavior of the soy-

bean trypsin inhibitor, especially in relation to the well-known effectiveness of methionine as a supplement to raw soybean meal.

#### EXPERIMENTAL

A trypsin inhibitor concentrate was prepared from solvent-extracted soybean flour<sup>4</sup> according to the procedure of Klose, Hill and Fevold ('46a). The antitryptic activity of this preparation was found to be approximately one-third that of the crystalline soybean trypsin inhibitor.<sup>5</sup>

The soybean hydrolysate was prepared by autoclaving solvent-extracted soybean flour with 4 parts of 9 N sulfuric acid at 15 lb. pressure for 18 hours. The sulfuric acid was removed with barium hydroxide to pH 4.2, and the resultant filtrate was concentrated *in vacuo* and lyophilized.

The autoclaved soybean meal used in this study was obtained by subjecting the solvent-extracted soybean flour to 15 lb. pressure for 20 min. (115°C.) while spread in the bottom of an enameled pan to a depth of about one inch. The autoclaved material was finely comminuted before being mixed into the appropriate diet.

The composition of the 4 basal diets employed in this study is presented in table 1. A protein level of 12% ( $N \times 6.25$ ) was chosen for the diets containing soybean protein because it has been shown that this level gives the maximum utilization of nitrogen for growth (Barnes, Maack, Knights and Burr, '45; Westfall and Hauge, '48). In the case of the hydrolysate, DL-tryptophan and L-cystine were added to compensate for possible losses incurred during acid hydrolysis; the amounts of each required to restore the original level of these amino acids in the unhydrolyzed protein were added. Also included in this study were diets containing casein provided at a level of 18% protein. The trypsin inhibitor preparation and DL-methionine, when used as adjuncts to the basal diets, were added without an equivalent deduction in protein in order

<sup>4</sup> Nutrisoy XXX, Archer-Daniels-Midland, Minneapolis, Minn.

<sup>5</sup> Generously provided by Dr. M. Kunitz, The Rockefeller Institute for Medical Research, Princeton, New Jersey.

to eliminate the possibility that any inhibiting effect would be even partly due to a lowered concentration of available amino acids in the diet.

Fifteen groups of 7 weanling male rats each (Sprague-Dawley) were placed on the various experimental diets when

TABLE 1

*Composition of basal diets used to demonstrate the effect of supplemental methionine in the presence of the soybean trypsin inhibitor*

INGREDIENT	DIET NUMBER			
	A	B	C	D
	%	%	%	%
Basic mix <sup>1</sup>	20.00	20.00	20.00	20.00
Nutrisoy XXX <sup>2</sup>	23.40			
Autoclaved Nutrisoy XXX <sup>3</sup>		23.85		
Soybean hydrolysate <sup>4</sup>			18.25	
L-cystine <sup>5</sup>			0.25	
DL-tryptophan <sup>6</sup>			0.15	
Casein, vitamin-free <sup>7</sup>				20.25
Corn starch	56.60	56.15	61.35	59.75

<sup>1</sup> Provides the following components per 100 gm diet: Wesson oil, 5.0 gm; salt mix, U.S.P. 2, 4.0 gm; sucrose, 9.0 gm; Wilson's 1-20 liver powder, 0.4 gm; riboflavin, 1.0 mg; thiamine, 0.5 mg; pyridoxine, 0.5 mg; niacin, 2.0 mg; calcium pantothenate, 2.5 mg; para-aminobenzoic acid, 25.0 mg; choline, 200 mg; inositol, 20.0 mg; 2-methyl-1, 4-naphthoquinone, 1.0 mg; biotin, 0.02 mg; alpha-tocopherol, 2.5 mg; haliver oil, 50 mg.

<sup>2</sup> Solvent-extracted soybean flour, equivalent to 12% protein based on N value of 8.21%.

<sup>3</sup> Equivalent to 12% protein based on N value of 8.05%.

<sup>4</sup> Equivalent to 11.7% protein based on N value of 10.25%.

<sup>5</sup> Equivalent to 0.18% protein and calculated to give a cystine value of 1.9 gm/16 gm N reported for soybean protein (Block and Mitchell, '46).

<sup>6</sup> Equivalent to 0.13% protein and calculated to give a tryptophan value of 1.2 gm/16 gm N reported for soybean protein (Block and Mitchell, '46).

<sup>7</sup> Equivalent to 18% protein based on N value of 14.21%.

they weighed 35 to 45 gm. The animals were put in individual cages provided with wire mesh bottoms. Food and water were provided ad libitum for a period of 16 days, during which time daily records were kept of individual food consumption and weight. The spillage of food was minimized by using small ointment jars placed in larger containers so that the whole



feeding apparatus could be weighed together, thus avoiding any loss of the small amounts of food spilled into the outer container. As a further precaution, sheets of paper were placed under each cage so that excessive amounts of spilled food could always be recovered.

The protein efficiency ratio (Osborne, Mendel and Ferry, '19), calculated as the grams gain in weight per gram of protein consumed, was employed as an index of the growth-promoting value of the various experimental diets.<sup>6</sup> These results are given in table 2. Apparent differences were evaluated by the "t" test (Snedecor, '46) as is shown in table 3, and only those showing a P value of less than 0.01 were considered significant. The reliability of protein-efficiency ratios determined over relatively short feeding periods has been pointed out by Klose, Greaves and Fevold ('48) and by Russell, Taylor, Mehrof and Hirsch ('46).

### RESULTS

It will be noted in table 2 that supplemental methionine improved the nutritive value of all diets, including those from which the trypsin inhibitor had been omitted. Thus any effect which supplemental methionine may have in conjunction with the trypsin inhibitor is obscured. In order to differentiate quantitatively between these two effects, the percentage increase in protein efficiency (PE) over that of the unsupplemented diets as a consequence of the addition of methionine was calculated. The relative effectiveness of supplemental methionine upon the growth-promoting value of the various diets could then be judged by the magnitude of the differences in percentage units.

<sup>6</sup> The authors recognize the limitations of this method of evaluating the nutritive value of dietary protein, which Mitchell ('44) has criticized on various grounds. The work of Barnes, Maack, Knights and Burr ('45), however, would appear to justify the use of the maximum PE ratio as a reliable index of the relative growth-promoting quality of dietary protein. Since much of the previous work relating to growth inhibitors derived from soybean has involved measurement of the gain in weight in relation to protein consumption, choice of this procedure by the authors was further governed by the desire to obtain data which would permit comparison with reported results in the literature.

It becomes apparent that methionine added to the raw soybean meal diet (compare groups 1 and 2) or to the autoclaved soybean meal diet containing the trypsin inhibitor (compare groups 5 and 6) was relatively more effective in promoting growth than when added to autoclaved soybean meal alone (compare groups 3 and 4). In the latter case, its relative in-

TABLE 2

*The effect of supplemental methionine on the nutritive value of diets containing a crude concentrate of the soybean trypsin inhibitor*

GROUP	DIET	PRO- TEIN <sup>1</sup>	AVG. GAIN IN WEIGHT	AVG. FOOD CON- SUMP- TION	AVG. PRO- TEIN CON- SUMP- TION	PROTEIN EFFICIENCY <sup>2</sup>	EFFECT- IVENESS OF SUPPLE- MENTAL METHI- ONINE <sup>3</sup>
		%	gm	gm	gm		%
1	Raw soybean	12.7	19.1	107.2	13.6	1.40 ± 0.15	
2	Raw soybean + 0.6% methionine	13.1	46.4	146.4	19.2	2.42 ± 0.10	74.0
3	Autoclaved soybean	12.5	61.6	187.0	23.4	2.63 ± 0.10	
4	Autoclaved soybean + 0.6% methionine	14.1	82.6	196.1	27.6	2.99 ± 0.03	13.7
5	Autoclaved soybean + 1.8% inhibitor	13.8	37.3	138.5	19.1	1.95 ± 0.09	
6	Autoclaved soybean + 1.8% inhibitor + 0.6% methionine	14.6	61.8	161.0	23.5	2.63 ± 0.07	34.8
7	Soybean hydrolysate	13.1	25.7	110.9	14.5	1.77 ± 0.06	
8	Soybean hydrolysate + 0.6% methionine	13.0	26.3	98.1	12.7	2.07 ± 0.05	16.9
9	Soybean hydrolysate + 1.8% inhibitor	13.6	21.6	103.0	14.0	1.54 ± 0.03	
10	Soybean hydrolysate + 1.8% inhibitor + 0.6% methionine	14.1	25.4	100.0	14.1	1.80 ± 0.04	16.8
11	Soybean hydrolysate + 1.8% autoclaved inhibitor <sup>4</sup>	13.7	23.7	94.5	12.9	1.84 ± 0.05	
12	18% casein	19.0	90.6	176.5	33.6	2.69 ± 0.06	11.9
13	18% casein + 0.6% methionine	19.3	92.3	158.5	30.6	3.01 ± 0.04	
14	18% casein + 1.8% inhibitor	20.2	76.7	163.3	33.0	2.32 ± 0.05	
15	18% casein + 1.8% inhibitor + 0.6% methionine	20.6	90.3	167.4	34.5	2.61 ± 0.08	12.5

<sup>1</sup> N × 6.25.

<sup>2</sup> Protein efficiency (grams gain in weight per gram protein consumed) determined over a feeding period of 16 days. The mean of each group (7 animals) ± the standard error of the mean.

<sup>3</sup> The per cent increase in protein efficiency when diet is supplemented with DL-methionine, calculated as follows:

$$\frac{\text{PE}(\text{diet} + \text{methionine}) - \text{PE}(\text{diet} - \text{methionine})}{\text{PE}(\text{diet} - \text{methionine})} \times 100.$$

<sup>4</sup> Autoclaved at 15 lb. for 20 min.

effectiveness can be adequately explained by the fact that methionine is the limiting amino acid of soybean protein even when properly heated (Almquist, Mecchi, Kratzer and Grau, '42). The greater efficacy of methionine with raw soybean or autoclaved soybean plus inhibitor suggests that a factor other than a deficiency of methionine is involved, which interferes

TABLE 3

*Statistical evaluation of protein efficiency ratios by the "t" test<sup>1</sup>*

GROUP NUMBER	COMPARED WITH GROUP NUMBER				
	Basal protein: Raw or autoclaved soybean protein				
	2	3	4	5	6
1	5.80	7.05	16.75	3.46	7.24
2		1.58	5.82	3.34	1.88
3			3.66	4.96	0.08
4				11.23	4.85
5					5.65
	Basal protein: Soybean hydrolysate				
	8	9	10	11	
7	3.56	6.48	1.26	0.78	
8		12.65	6.18	3.29	
9			4.06	7.81	
10				2.57	
	Basal protein: "18%" casein				
		13	14	15	
12		3.85	4.15	0.65	
13			9.65	4.05	
14				2.90	

<sup>1</sup> Calculated according to Snedecor ('46). Only those "t" values greater than 3.055 ( $P < .01$ ) were considered indicative of significant differences.

with the availability of methionine for growth. This is not the whole explanation, however. If the only effect of the inhibitor were to decrease the availability of methionine, then adding an excess of methionine to either soybean (group 2) or to autoclaved soybean plus inhibitor (group 6) should elevate their PE to the same level as that of methionine-supplemented autoclaved soybean (group 4). This disparity, which persists in spite of the adequacy of methionine, is indicative

of still another factor, not involving the availability of methionine, which adversely affects growth.

The lower PE values obtained for the diets containing the soybean hydrolysate may be attributed to the inferiority of hydrolyzed soybean protein to unhydrolyzed protein in respect to maintenance of a positive N balance which was observed by Supplee and Clark ('46). In contrast to the results observed with the intact soybean protein, the improvement in the nutritive value of the diet containing soybean hydrolysate and inhibitor as a result of supplementation with methionine (compare groups 9 and 10) was not appreciably different from the effect produced by adding methionine to the hydrolysate alone (compare groups 7 and 8). In this instance, therefore, the beneficial effect of methionine could be essentially accounted for by the fact that the hydrolysate was deficient in this amino acid. The difference in PE between the diet containing methionine-supplemented hydrolysate (group 8) and methionine-supplemented hydrolysate plus inhibitor (group 10) is again attributable to a mechanism totally unrelated to the utilization of methionine for growth. The additional protein furnished by the trypsin inhibitor preparation did not significantly affect the PE of the basal diet (compare groups 7 and 11).

It was thought that the incorporation of casein at an 18% protein level would provide a diet in which the effectiveness of methionine in relation to the inhibitor could be studied directly, without the complication of a methionine deficiency. The data in table 2, however, show that the 18% casein diet could be further supplemented with methionine. Subsequent examination of the literature revealed that at least one paper has reported the same observation (Mulford and Griffith, '42). Data relating to the relative effectiveness of supplemental methionine are similar to the results obtained with the soybean hydrolysate. As in the case of the latter, the effect of supplemental methionine on the diets containing casein and

the inhibitor (compare groups 14 and 15)<sup>7</sup> was about the same as would be expected to result from the addition of methionine to diets containing casein alone (compare groups 12 and 13). Consistent with the data obtained with intact and hydrolyzed soybean protein is the observation that the diet containing 18% casein plus inhibitor (group 15) remained nutritionally inferior to a similar diet from which the inhibitor had been omitted (group 13) even when both diets were adequately supplemented with methionine (see table 3, last column, "t" = 4.05).

#### DISCUSSION

On the basis of the foregoing evidence, it becomes necessary to postulate that the mode of action of concentrates of the trypsin inhibitor with respect to growth may involve two mechanisms: (A) one which interferes with the availability of methionine for growth; and (B) one which exerts a growth-depressing effect through a mechanism unrelated to A. When the source of N in the diet is intact soybean protein, it appears that both mechanisms A and B are responsible for growth inhibition; when the N is contributed by hydrolyzed soybean protein, mechanism B is apparently the only causative factor involved. Since mechanism A did not come into play when the protein had been subjected to complete hydrolysis, it is probable that this mechanism depends on the anti-proteolytic action of the inhibitor. Similarly, mechanism B does not depend on an inhibition of proteolysis. Since a crude concentrate of the trypsin inhibitor was used in this study, it cannot be inferred that mechanisms A and B are necessarily caused by the same substances.

The demonstration by Hayward and Hafner ('41), Almquist, Mecchi, Kratzer and Grau ('42), and others that the addition of methionine to raw soybean meal improves growth to a greater extent than when it is added to autoclaved soybean meal has been interpreted to indicate that heating in-

<sup>7</sup> The effectiveness of supplemental methionine in this instance was not quite significant at the 1% level. See table 3.

creases the availability of methionine from soy protein. The data presented here substantiate this view and suggest further that it is the trypsin inhibitor of unheated soybean meal which interferes with the availability of methionine for growth. It has, however, never been entirely clear why the nutritive value of raw and autoclaved soybean meal should not be equalized when both are supplemented with an excess of methionine. Hayward and Hafner ('41) have suggested that autoclaving soybean meal raises its general plane of nutrition by increasing the availability of other amino acids as well, which might have been the limiting factor for growth in the case of the unheated meal even in the presence of adequate amounts of supplemental methionine. The absence of other limiting amino acids in raw soybean meal, however, has been demonstrated by Klose, Hill and Fevold ('46b). These workers showed that methionine alone was just as effective a supplement to raw soybean meal as all 10 essential amino acids. It appears quite likely, therefore, that this discrepancy between raw and autoclaved soybean meal when both are supplemented with methionine can be more simply explained by the hypothesis that the trypsin inhibitor (or some associated substance) exerts a separate growth-retarding effect, designated herein as mechanism B. This same effect could very well account for the ability of active trypsin inhibitor preparations to inhibit the growth of animals receiving diets containing protein hydrolysates.

Although the crude trypsin inhibitor preparation depressed the growth of rats receiving a diet containing 18% casein, this inhibition did not appear to involve any interference with the availability of methionine for growth, as judged by the effectiveness of supplemental methionine. If mechanism B, therefore, is the only factor responsible for the adverse effect of the trypsin inhibitor on the nutritive value of diets containing casein, it must follow that the inhibitor will (1) have the same quantitative effect on either casein or its hydrolysate, and (2) retard growth to a greater extent when added to diets containing properly heated soybean protein, where

both mechanisms A and B operate, then when incorporated into diets containing casein. Positive evidence for both of these points is at hand. With respect to (1), Klose, Greaves and Fevold ('48) and Westfall, Bosshardt, and Barnes ('48) found that trypsin inhibitor preparations depressed the growth of rats on diets containing casein hydrolysates to at least the same extent as they did the growth of those fed unhydrolyzed casein. Regarding (2), Westfall and Hauge ('48) found that the deleterious effect of the inhibitor on the nutritive value of casein was less than on properly heated soybean flour. Similarly, Ham, Sandstedt and Mussehl ('45) observed that a trypsin inhibitor prepared by acetone precipitation from aqueous extracts of raw soybean exerted a greater inhibitory effect on the growth of chicks fed autoclaved soybean meal than on the growth of those fed protein of animal origin.

Recent papers (Riesen, Clandinin, Elvehjem and Cravens, '47; Hou, Riesen and Elvehjem, '49; Liener and Fevold, '49) have shown that the rate of release of methionine remains an inadequate explanation for the poor nutritive value of raw soybean. Nevertheless, the observation that the interference with the availability of methionine exerted by the trypsin inhibitor depends on its antiproteolytic activity invites a re-evaluation of the concept relating to inhibition of the rate of release of methionine (Melnick, Oser and Weiss, '46). If one considers the rate of intestinal absorption of the amino acids (Wilson and Lewis, '29; Chase and Lewis, '33) as well as their rate of liberation, the mutual supplementation of these acids could be expected to play a significant role in their utilization by the animal organism, in the following manner. If, in unheated soybean meal, the rate of proteolytic digestion is lower than the absorptive capacity of the intestine, there would be no tendency for amino acids to accumulate in the intestinal tract, and the concentration of the limiting amino acid, methionine, may be insufficient to permit adequate supplementation of the other amino acids. On the other hand, if, after the meal has been heated to inactivate the trypsin

inhibitor, the rate of digestion is higher than the intestinal absorptive capacity, the digestion products would tend to accumulate, and the methionine concentration may be great enough to act in a mutually supplementary manner with the other amino acids which are in excess. In the case of proteins of animal origin such as casein, in which methionine is relatively more abundant,<sup>8</sup> it is possible that the differential between the rate of proteolytic digestion, as affected by the trypsin inhibitor, and the rate of intestinal absorption may not be great enough to depress the concentration of methionine to the point where it will not adequately supplement the other amino acids. Under such conditions, the ability of the trypsin inhibitor to interfere with the availability of methionine would not play a significant role in the over-all effect of the inhibitor on the nutritive value of casein and other proteins in which a deficiency of methionine is not as critical as in the case of soybean protein.

#### SUMMARY

Animal experiments involving measurement of the growth performance of rats were conducted to determine the effect of supplemental methionine on diets containing a soybean trypsin inhibitor concentrate and in which the sources of nitrogen were raw or autoclaved soybean protein, soybean protein hydrolysate, or casein. When the soybean hydrolysate or casein was furnished in the diet, the depression in growth exerted by the trypsin inhibitor preparation was not related to interference with the availability of methionine for growth. The beneficial effect of methionine in this instance could be wholly accounted for by the fact that the hydrolysate and casein were deficient to some extent in this amino acid. With diets containing autoclaved soybean meal, the growth-retarding action of the inhibitor was referable to interference with the availability of methionine for growth, as well as to the deficiency observed in the case of the hydrolysate and casein.

<sup>8</sup> Block and Mitchell ('46) report the methionine content of casein and soybean protein to be 3.5 and 2.0 gm/16 gm N, respectively.



These results may be explained by postulating a two-fold mode of action by active trypsin inhibitor preparations, one which results in an impairment of the availability of methionine for growth as a consequence of its antiproteolytic activity, and the other having an effect unrelated to the inhibition of proteolysis. The former can be demonstrated if the source of ingested N is an intact protein critically deficient in methionine. The latter effect is independent of the nature and quality of ingested N.

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# THE EFFECT OF CARBOHYDRATE ON THE NUTRITIVE VALUE OF HEATED LACTALBUMIN<sup>1</sup>

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## THREE FIGURES

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It is generally accepted that the nutritive value of a protein depends essentially upon the kinds of amino acids which it contains and upon the quantitative proportion of each which is absorbed at a given time. Physical and chemical factors which destroy the amino acids or alter their availability to the organism will therefore affect the nutritional quality of the protein. Numerous investigations have been undertaken in an attempt to evaluate the effect of heat on proteins. These studies date from the work of McCollum and Davis ('15), who were probably the first to report that the protein constituent of milk is responsible for the loss in nutritive value which occurs when this fluid is heated. Fairbanks and Mitchell ('35) and Mumford ('33) also observed the sensitivity of milk proteins to heat. In addition, heating has been shown to produce detrimental effects on the nutritive value of beef protein (Morgan and Kern, '34; Seegers and Mattill, '35), fish meal (Schneider, '32; Maynard, Bender and McCay, '32), human globin (Devlin and Zittle, '44), field peas (Woods, Beeson and Bolin, '43), edestin (Waisman and Elvehjem, '38), pea-

<sup>1</sup>The data in this paper are taken from the dissertation submitted by Ivan J. Mader for the degree of Master of Science, Wayne University, 1949. A preliminary report of the work was given before the American Institute of Nutrition at Detroit, April 19, 1949.

nuts (Mitchell, Burroughs and Beadles, '36), cottonseed meal (Olcott and Fontaine, '41), gluten (Morgan and Petro, '30), soybean oil meal (Evans, McGinnis and St. John, '47), cereal proteins (Murlin, Nasset and Marsh, '38; Kuether and Myers, '48), and lactalbumin (Davis, Rizzo and Smith, '49).

Proteins and carbohydrates are known to interact under a variety of conditions. In 1912 Maillard described a reaction between amino acids and reducing sugars which formed humin-like substances, "melanoidins." This original observation was confirmed by Borsook and Wasteneys ('25), who reported a reduction of free amino nitrogen after an interaction between glucose and protein derivatives. The Maillard or melanoidin condensation theory is the classical explanation of non-enzymatic browning in food products (Stadtman, '48). The extent of the reaction appears to be in proportion to the availability of free aldehyde groups (Ransey, Tracy and Ruehe, '33; Ross, '48) and amino groups (Patton and Pyke, '46).

The effect of the browning reaction on the nutritive value of microbiological media has been investigated by Patton and Hill ('48), and Hill and Patton ('47), who reported the destruction of certain essential amino acids resulting from an interaction between the protein and reducing sugar components of the media. These results are in accord with those of Riesen et al. ('47), who studied the liberation of essential amino acids from heated soybean oil meal.

The widespread occurrence of the browning reaction and the suggestion that the interaction of carbohydrates and proteins is responsible for the nutritive damage which occurs when protein is heated, have prompted a further study of these effects in relation to the mammalian species. The present investigation has demonstrated the low nutritive value of heated protein for the adult dog and has described the effect of carbohydrate, when heated with the protein, on the nutritive value of the protein. The *in vivo* studies have been evaluated in terms of the reducing sugar content of the preparations used.

## EXPERIMENTAL

Lactalbumin<sup>2</sup> was the protein used for all experiments. This protein will hereafter be referred to as "commercial lactalbumin." Preliminary analysis showed that it contained relatively large amounts of reducing substances which could be easily extracted with water. The following procedure was used to prepare the product hereinafter termed "extracted lactalbumin."

Two kilograms of commercial lactalbumin were suspended in 5 l of distilled water and vigorously stirred mechanically for 30 min. The protein was recovered by centrifugation and resuspended in 5 l of water. This alternate washing and centrifugation operation was carried out 8 times over a period of three successive days. The protein was then washed in 5 l of 5% ethanol for 30 min., was centrifuged, washed finally with distilled water, again centrifuged, and allowed to dry in air. Thus, the commercial lactalbumin was extracted with 20 to 25 volumes of fluid. The extracted lactalbumin did not give a Molisch test.

Heat treatment of the commercial and extracted proteins included dry-heating or autoclaving, or both, for varying periods of time. For the dry-heating process, the lactalbumin was placed in shallow pans in layers not over 1 cm deep and baked in an oven at 120°C. for 60 min. When the protein was autoclaved it was placed in shallow pans and heated at 120°C. (15 lb. pressure) for 30 or 60 min. After autoclaving, the material was cooled rapidly, spread out in a thin layer and allowed to air-dry for at least three days before being used.

A purified ration containing all the known dietary essentials was prepared. The percentage composition of this basic diet, patterned after that used by Orten and Smith ('37) was: lactalbumin 10.0, sucrose 40.0, white dextrin 10.0, commercial hydrogenated cottonseed oil<sup>3</sup> 25.0, dried yeast<sup>4</sup> 6.8, Wesson salt mixture 3.5, bone ash 2.7, cod liver oil 2.0. The protein

<sup>2</sup> Lactalbumin 15-42, obtained from the Borden Company.

<sup>3</sup> Crisco.

<sup>4</sup> Anheuser-Busch, Strain G.

component of the diet was adjusted for all control and experimental periods to insure each animal's consumption of the same amount of nitrogen every day. Sucrose was correspondingly increased or decreased to make all rations isocaloric.

Three healthy adult female dogs, each weighing approximately 10 kg, were used as experimental subjects. They were kept in individual standard metabolism cages and had access to water at all times. The animals were fed at the same hour daily and weighed weekly. They ate their ration promptly and completely at all times.

The method employed for evaluating the nutritional quality of the proteins was adapted from the one used by Murlin and his associates ('41). This procedure involved a comparison of the amount of urinary and fecal nitrogen excreted by a subject fed a diet containing a "control" or reference protein with the amount excreted by the same subject when fed a similar diet containing the protein to be tested in place of the "control" protein. In the present study either commercial or extracted lactalbumin, unheated, served as the reference protein to which the heated proteins were compared. The experiment consisted of alternate control periods, each three days in length, and experimental periods of 4 days. The protein to be tested was fed for two 4-day periods separated by a control period.

Urine samples from each dog were obtained quantitatively by catheterization for all collection periods and were preserved with sulfuric acid and toluene. The urine was made up to volume and aliquots were analyzed for nitrogen. The fresh feces, separated into periods with carmine, were kept covered with ethanol containing 1% sulfuric acid until dried on a steam cone. Nitrogen determinations were made on aliquots of the ground dried material. All analyses for nitrogen were made in replicate by the macro-Kjeldahl method.

The caloric requirement of each dog was first determined with a diet containing 10% casein. That amount of ration which precluded any gain or loss in body weight was fed to the dog in all succeeding studies. A daily ration furnishing



45 to 75 cal. per kilogram of body weight was found to be satisfactory, depending upon the individual dog. The maximum weight change in any dog throughout the experiment was only 0.7 kg.

Before studying the effect of heat on the nutritive value of the lactalbumin it was desirable to conduct a number of balance studies which would determine the minimum amount of

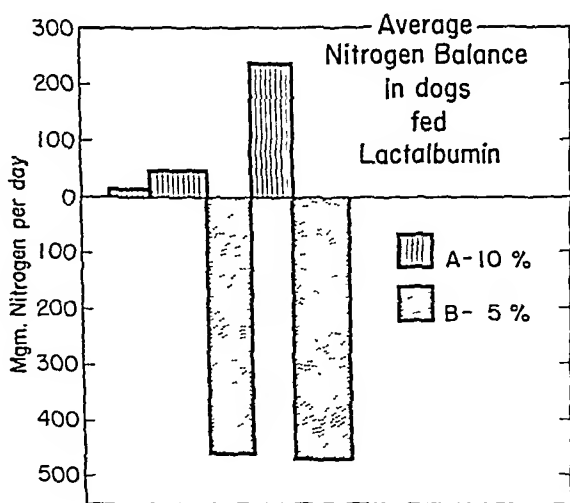


Fig. 1 Preliminary nitrogen balance studies to determine the minimum nitrogen requirement of the dogs.

protein necessary to keep the dogs in nitrogen balance. Accordingly, diets containing 5 and 10% of commercial lactalbumin were prepared and fed to the dogs in an amount commensurate with their caloric requirements. The averages of the nitrogen balances of the animals maintained on these diets are shown in figure 1. It is evident that lactalbumin fed at a level of 10% was adequate for nitrogen balance in the present study. The yeast of the diet supplied an appreciable, and constant, amount of nitrogen. This nitrogen comprised approximately 0.4% of the diet. There was a total dietary nitrogen content of about 1.67%, and all other diets contain-

ing a treated protein were constituted to contain as nearly as possible this amount of nitrogen.

Complete nitrogen balance data on each dog for all control and experimental periods are presented in table 1. Figures 2 and 3 show in chronological sequence the averages of the

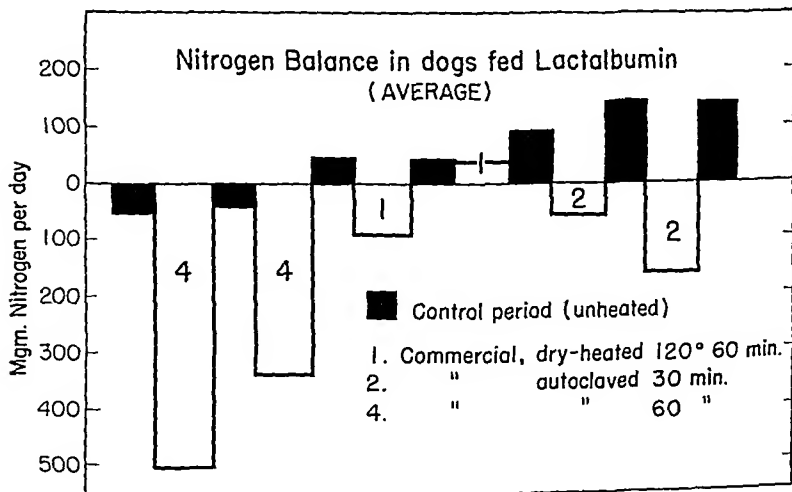


Fig. 2 Average nitrogen balances in dogs fed unextracted (commercial) lactalbumin.

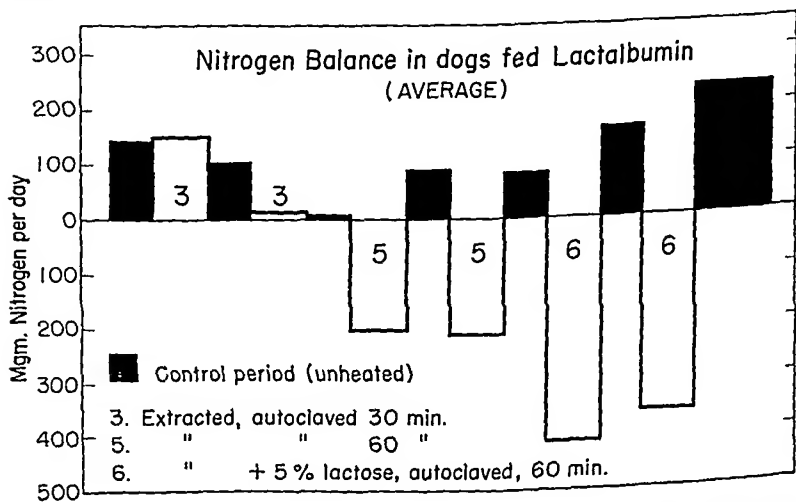


Fig. 3 Average nitrogen balances in dogs fed extracted (carbohydrate-free) lactalbumin.

TABLE 1  
Food, urinary and fecal nitrogen data

	TOTAL FOOD N			TOTAL URINARY N			TOTAL FECAL N		
	Dog No.		3	Dog No.		3	Dog No.		3
	1	2		1	2		1	2	
	gm			gm			gm		
Commercial lactalbumin, unheated <sup>1</sup>	6.26	6.26	4.51	5.11	5.84	3.69	1.04	0.96	0.86
Commercial lactalbumin, autoclaved 60 min. <sup>2</sup>	8.15	8.15	5.25	6.16	6.83	4.78	4.03	2.98	2.97
Commercial lactalbumin, unheated	6.26	6.26	4.51	5.38	5.79	3.72	0.95	0.77	0.80
Commercial lactalbumin, autoclaved 60 min.	8.15	8.15	5.87	5.67	6.70	4.61	4.29	2.05	2.93
Commercial lactalbumin, unheated	6.26	6.26	4.51	4.55	5.74	3.44	1.05	0.96	0.86
Commercial lactalbumin, dry-heated 120°C. 60 min.	7.75	7.75	5.58	5.80	7.09	4.58	1.78	1.37	1.60
Commercial lactalbumin, unheated	6.34	6.34	4.56	4.89	5.72	3.51	1.01	0.87	0.86
Commercial lactalbumin, dry-heated 120°C. 60 min.	7.75	7.75	5.58	5.61	5.71	4.42	1.68	1.68	1.54
Commercial lactalbumin, unheated	6.38	6.38	4.59	4.78	5.12	3.67	1.04	1.02	0.83
Commercial lactalbumin, autoclaved 30 min.	8.25	8.25	5.94	5.50	6.05	4.75	3.33	2.37	1.19
Commercial lactalbumin, unheated	6.38	6.38	4.59	4.47	5.21	3.46	1.08	1.03	0.84
Commercial lactalbumin, autoclaved 30 min.	8.25	8.25	5.94	5.35	6.36	4.67	2.86	2.74	2.50
Commercial lactalbumin, unheated	6.38	6.38	4.59	4.58	5.21	3.49	1.07	0.84	0.92
Extracted lactalbumin, autoclaved 30 min.	8.25	8.25	5.94	5.70	5.93	4.58	1.56	1.44	1.45
Extracted lactalbumin, unheated	6.15	6.15	4.43	4.55	5.10	3.44	1.06	0.83	0.83
Extracted lactalbumin, autoclaved 30 min.	8.25	8.25	5.94	6.01	6.83	5.24	1.45	1.47	1.26
Extracted lactalbumin, unheated	6.15	6.15	4.43	4.73	5.27	3.98	1.11	0.71	0.83
Extracted lactalbumin, autoclaved 60 min.	8.20	8.20	5.90	5.84	5.84	4.87	2.90	2.73	2.57
Extracted lactalbumin, unheated	6.15	6.15	4.43	4.78	5.18	3.29	1.02	0.80	0.87
Extracted lactalbumin, autoclaved 60 min.	8.04	8.04	5.85	5.67	6.47	4.52	3.02	2.42	2.40
Extracted lactalbumin, unheated	6.15	6.15	4.43	4.55	5.22	3.41	0.96	0.93	0.91
Extracted lactalbumin + 5% lactose, autoclaved 60 min.	8.10	8.10	5.83	4.78	5.84	4.44	4.82	3.85	3.25
Extracted lactalbumin, unheated + 5% lactose	6.34	6.34	4.56	4.41	5.26	3.26	0.97	0.91	0.97
Extracted lactalbumin + 5% lactose, autoclaved 60 min. <sup>3</sup>	6.07	6.07	4.37	3.44	3.84	3.27	3.43	3.18	2.60
Extracted lactalbumin, unheated + 5% lactose <sup>4</sup>	12.67	12.67	9.13	7.53	9.97	6.44	2.27	2.30	1.74

<sup>1</sup> Each unheated protein was fed for a period of three days.

<sup>2</sup> Each heated protein was fed for a period of 4 days.

<sup>3</sup> Three-day period.

<sup>4</sup> Six-day period.

ing a treated protein were constituted to contain as nearly as possible this amount of nitrogen.

Complete nitrogen balance data on each dog for all control and experimental periods are presented in table 1. Figures 2 and 3 show in chronological sequence the averages of the

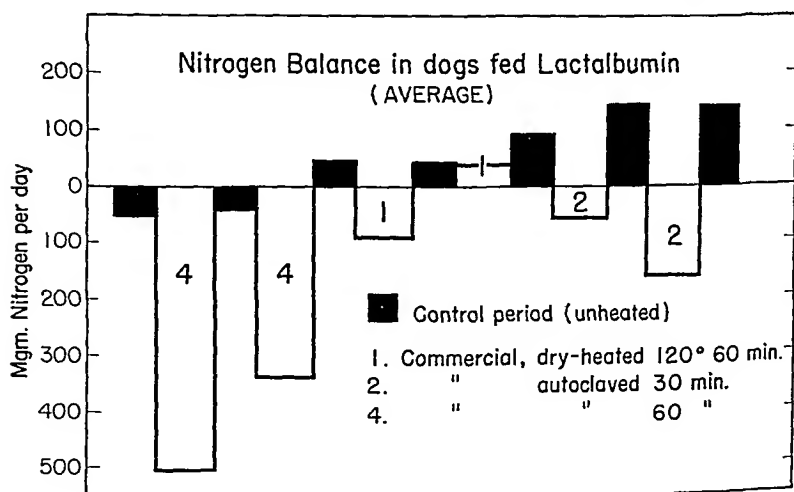


Fig. 2 Average nitrogen balances in dogs fed unextracted (commercial) lactalbumin.

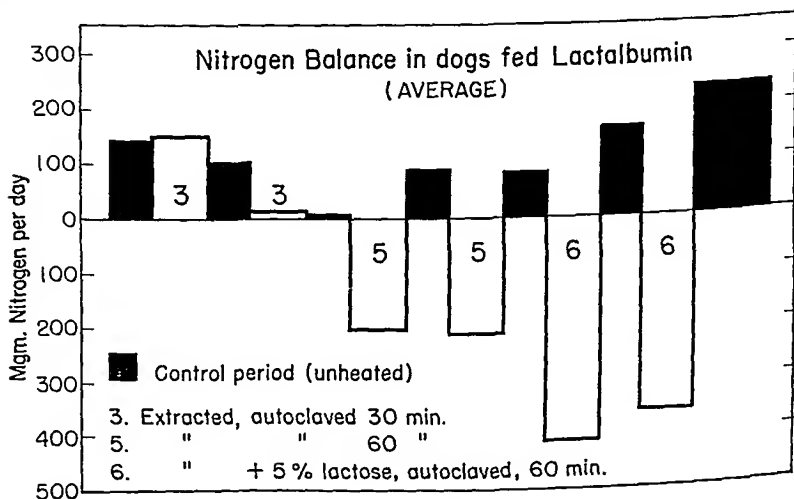


Fig. 3 Average nitrogen balances in dogs fed extracted (carbohydrate-free) lactalbumin.

TABLE 1  
*Food, urinary and fecal nitrogen data*

	TOTAL FOOD N			TOTAL URINARY N			TOTAL FECAL N		
	Dog No.			Dog No.			Dog No.		
	1	2	3	1	2	3	1	2	3
	<i>gm</i>			<i>gm</i>			<i>gm</i>		
Commercial lactalbumin, unheated <sup>1</sup>	6.26	6.26	4.51	5.11	5.84	3.69	1.04	0.96	0.86
Commercial lactalbumin, autoclaved 60 min. <sup>2</sup>	8.15	8.15	5.25	6.16	6.83	4.78	4.03	2.98	2.87
Commercial lactalbumin, unheated	6.26	6.26	4.51	5.38	5.79	3.72	0.95	0.77	0.80
Commercial lactalbumin, autoclaved 60 min.	8.15	8.15	5.87	5.67	6.70	4.61	4.29	2.05	2.93
Commercial lactalbumin, unheated	6.26	6.26	4.51	4.55	5.74	3.44	1.05	0.96	0.86
Commercial lactalbumin, dry-heated 120°C. 60 min.	7.75	7.75	5.58	5.80	7.09	4.58	1.78	1.37	1.60
Commercial lactalbumin, unheated	6.34	6.34	4.56	4.89	5.72	3.51	1.01	0.87	0.86
Commercial lactalbumin, dry-heated 120°C. 60 min.	7.75	7.75	5.58	5.61	5.71	4.42	1.68	1.68	1.54
Commercial lactalbumin, unheated	6.38	6.38	4.59	4.78	5.12	3.67	1.04	1.02	0.88
Commercial lactalbumin, autoclaved 30 min.	8.25	8.25	5.94	5.50	6.05	4.75	3.33	2.37	1.19
Commercial lactalbumin, unheated	6.38	6.38	4.59	4.47	5.21	3.46	1.08	1.03	0.84
Commercial lactalbumin, autoclaved 30 min.	8.25	8.25	5.94	5.35	6.36	4.67	2.86	2.74	2.50
Commercial lactalbumin, unheated	6.38	6.38	4.59	4.58	5.21	3.49	1.07	0.84	0.92
Extracted lactalbumin, autoclaved 30 min.	8.25	8.25	5.94	5.70	5.93	4.58	1.56	1.44	1.45
Extracted lactalbumin, unheated	6.15	6.15	4.43	4.55	5.10	3.44	1.06	0.83	0.83
Extracted lactalbumin, autoclaved 30 min.	8.25	8.25	5.94	6.01	6.83	5.24	1.45	1.47	1.26
Extracted lactalbumin, unheated	6.15	6.15	4.43	4.73	5.27	3.98	1.11	0.71	0.83
Extracted lactalbumin, autoclaved 60 min.	8.20	8.20	5.90	5.84	5.84	4.87	2.90	2.73	2.57
Extracted lactalbumin, unheated	6.15	6.15	4.43	4.78	5.18	3.29	1.02	0.80	0.87
Extracted lactalbumin, autoclaved 60 min.	8.04	8.04	5.85	5.67	6.47	4.52	3.02	2.42	2.40
Extracted lactalbumin, unheated	6.15	6.15	4.43	4.55	5.22	3.41	0.96	0.93	0.91
Extracted lactalbumin + 5% lactose, autoclaved 60 min.	8.10	8.10	5.83	4.78	5.84	4.44	4.82	3.85	3.25
Extracted lactalbumin, unheated + 5% lactose	6.34	6.34	4.56	4.41	5.26	3.26	0.97	0.91	0.97
Extracted lactalbumin + 5% lactose, autoclaved 60 min. <sup>3</sup>	6.07	6.07	4.37	3.44	3.84	3.27	3.43	3.18	2.60
Extracted lactalbumin, unheated + 5% lactose <sup>4</sup>	12.67	12.67	9.13	7.53	9.97	6.44	2.27	2.30	1.74

<sup>1</sup> Each unheated protein was fed for a period of three days.

<sup>2</sup> Each heated protein was fed for a period of 4 days.

<sup>3</sup> Three-day period.

<sup>4</sup> Six-day period.

nitrogen balances of the three dogs as they were alternately fed the unheated and heat-treated proteins. Although there are individual variations in the extent of nitrogen balance, the direction of the change in nitrogen equilibrium for each dog is the same in all cases.

For calculating the digestibility and biological value of the heated proteins, a modification of the method proposed by Murlin et al. ('41) was used. It was assumed that the absorption and utilization of the control (unheated) lactalbumin is complete. The calculated digestibility and utilization are based, therefore, on unheated protein as representing a value of 100%. By multiplying the biological value by the digestibility, a quantitative expression is obtained which Cahill, Schroeder and Smith ('44) have termed the "nutritive index." This value expresses more accurately the over-all nutritive value of a protein than does either the digestibility or biological value alone. For each heated protein fed, the averages of the data of the two experimental periods have been used for purposes of calculation. The averages of the data from the three control periods which delimit and separate the experimental periods have been similarly used.

In table 2 are listed the average per cent nitrogen output in the feces and the calculated digestibilities of all the heated proteins. It is apparent that the extent of absorption of the nitrogen of the heated proteins depends upon the method of heating and upon the length of the heating period. Dry-heating of commercial lactalbumin lowers the availability of the protein nitrogen only slightly, whereas if it is autoclaved the digestibility is markedly diminished. This decrease is directly proportional to the length of the heating period. Of special significance is the fact that the digestibility of autoclaved extracted lactalbumin was considerably higher than that of the autoclaved unextracted product. The absorption of the 30-min. autoclaved extracted protein was almost as complete as that of the unheated materials.

The average biological values and nutritive indices are shown in table 2. It will be noted that in all cases the utiliza-

tion of the absorbed nitrogen from the heated protein was as good as or better than that from the unheated protein. It is evident that no loss in nutritional quality was caused by the dry-heating process or by autoclaving extracted lactalbumin for 30 min. On the other hand, if commercial lactalbumin is autoclaved for 30 min. the nutritive index, which

TABLE 2

*Digestibility, fecal nitrogen, biological value and sugar content of heated lactalbumin as related to nutritive index*

	DIGESTIBILITY <sup>1</sup>	NITROGEN OUTPUT IN FECES		BIO- LOGICAL VALUE <sup>2</sup>	NUTRI- TIVE INDEX <sup>3</sup>	SUGAR CONTENT
		Control	Exptl.			
	%	%	%	%	%	%
Commercial lactalbumin, dry-heated 120°C., 60 min.	95	17	23	109	103	4.0
Commercial lactalbumin, autoclaved 30 min.	84	18	31	107	90	1.2
Extracted lactalbumin, autoclaved 30 min.	97	17	20	104	100	0.3
Commercial lactalbumin, autoclaved 60 min.	72	16	36	111	80	0.8
Extracted lactalbumin, autoclaved 60 min.	80	17	33	107	85	0.3
Extracted lactalbumin + 5% lactose, autoclaved 60 min.	63	19	45	115	73	0.9

$$^1 \text{Digestibility (per cent)} = \frac{\text{Food N absorbed}}{\text{Total food N}} \times 100.$$

$$\text{Food N absorbed} = \text{Total food N} - \left( \begin{array}{c} \text{Experimental} \\ \text{fecal N} \end{array} - \begin{array}{c} \text{Control} \\ \text{fecal N} \end{array} \right).$$

$$^2 \text{Biological value (per cent)} = \frac{\text{Food N absorbed} - \left( \begin{array}{c} \text{Experimental} \\ \text{urinary N} \end{array} - \begin{array}{c} \text{Control} \\ \text{urinary N} \end{array} \right)}{\text{Food N absorbed}} \times 100.$$

$$^3 \text{Nutritive index} = \text{biological value} \times \text{digestibility}.$$

involves the factor of digestibility, is lowered by 10%. When the autoclaving period is extended to 60 min. the damage incurred by both commercial and extracted lactalbumin is considerable, though again the extracted protein is not so severely injured as is the commercial product with its higher content of carbohydrate.

It was shown in the balance study that the removal of reducing substances from commercial lactalbumin prior to the heating process markedly affects the nutritive value of the protein. It was therefore desirable to determine quantitatively the reducing sugar content of all the proteins used, in an attempt to correlate these results with the *in vivo* studies. A modification of the Shaffer and Hartmann copper reduction iodometric method for the determination of lactose in milk was used (Winton and Winton, '45). The results of these analyses (table 2) show that the recovery of lactose from commercial lactalbumin is markedly reduced after it has been autoclaved. The sugar content of the protein is decreased approximately 75 to 85% by autoclaving the material for 30 and 60 min., whereas the dry-heating process removes only 20% of the sugar. The marked loss of reducing sugar content is not due to the heat treatment per se, for if lactose is autoclaved with a finely divided inert material, such as talc, about 85% of the sugar is still found to be present after autoclaving. This suggests that a reaction between the carbohydrate and the protein has occurred.

#### DISCUSSION

The ability of lactalbumin to maintain nitrogen balance in the adult dog is impaired when the protein has been heated. This is in accord with the findings of many investigators who have used other proteins and different species of experimental animals. It has been reported that there is greater damage to the nutritive value of a protein as the length of the heating period is increased (Morgan and Kern, '34; Seegers and Mattill, '35; Davis, Rizzo and Smith, '49). This has been found to be true in the present study. It appears that the detrimental effect of heat is gradual and progressive.

Moisture and temperature increases accelerate the changes which occur in the protein as it is heated. In the present study, dry-heating of commercial lactalbumin produced very little alteration in the protein, judging from the nutritive in-



dex, whereas autoclaved protein was markedly inferior even when the duration of the heat treatment was less.

Recent work has shown that the reaction between reducing sugars and protein may be responsible for the decreased nutritive value of heated proteins (Patton et al., '48a, '48b; Hill and Patton, '47; Stevens and McGinnis, '47). The results of the present investigation support this hypothesis. Heated extracted lactalbumin differs markedly from heated commercial lactalbumin in its ability to maintain nitrogen balance in the adult dog. Whereas autoclaving the commercial product for 30 min. produces a material which is unable to support nitrogen equilibrium, the same treatment of extracted lactalbumin causes no apparent change as regards nitrogen availability. If the duration of heat treatment is increased to 60 min. the extracted protein becomes less adequate but its nutritive value is nevertheless greater than that of commercial lactalbumin subjected to the same treatment.

A critical analysis of the data shows that a change in the digestibility of the heated proteins can account for a major portion of the damage incurred. Morgan and co-workers ('26, '34) reported that the heating process does not affect the digestibility of a protein, though its biological value may be impaired. More recently Weast, Groody and Morgan ('48) observed that heated casein is not well absorbed by the growing dog. This is in direct contrast to the report by Morgan ('31) of large urinary nitrogen losses in rats fed heated protein. Others have found, however, that in this species the digestibility of heated protein is not as complete as that of unheated protein (Chick et al., '35; Davis, Rizzo and Smith, '49).

Paradoxically, the real biological value of all proteins used in the present experiments was uniformly high, indicating that whatever nitrogen the dogs were able to absorb was well utilized. Apparently the animals neither absorbed atypical peptides which were excreted in the urine, nor wasted absorbed nitrogen because some essential amino acid was

lacking, thereby preventing mutual simultaneous supplementation.

Our observations with respect to nitrogen balances and the carbohydrate content of the various proteins support the hypothesis that the browning reaction which occurs when protein is heated is some type of condensation between nitrogenous compounds and reducing sugars, specifically involving the amino groups of the protein and the free aldehyde groups of the carbohydrate. While the presence of carbohydrate in commercial lactalbumin has been shown to play a prominent role with regard to the nutritive value of the protein when it is subjected to heat treatment, it should be emphasized that this phenomenon is probably but one of many complex and little understood reactions which occur. Actual destruction of amino acids when casein or soybean oil meal is heated in the presence of carbohydrate has been reported (Patton et al., '48a, '48b). The heat treatment itself undoubtedly causes changes in the protein molecule, especially at high temperatures (Mecham and Olcott, '47). Decomposition of proteins becomes extensive at temperatures of 150°C. or more, with a loss of considerable amounts of ammonia, decreases in amino and basic groups, and changes in solubility.

It would appear, nevertheless, that an interaction of a protein molecule and a reducing sugar can account for much of the damage which occurs when commercial lactalbumin is heated. This reaction is particularly noticeable at relatively low temperatures and with comparatively short periods of heating. As the duration of heating is prolonged or the temperature increased, other reactions obscure this phenomenon.

#### SUMMARY

No change in its nutritional quality occurred when commercial lactalbumin was dry-heated at 120°C. for 60 min. Its digestibility and nutritive index were found to be significantly decreased by autoclaving. This decrease in nutritive value was in proportion to the severity of the heat

treatment. The nutritional quality of extracted lactalbumin was not as seriously impaired by comparable heat treatment. The latter was reconstituted with lactose with respect to the carbohydrate content of the original protein. Upon heating, the reducing sugar content diminished and severe negative nitrogen balances resulted. Decreased digestibility, as evidenced by increased fecal nitrogen, accounted for the decrease in nutritive value. Utilization of absorbed nitrogen remained uniformly high in all cases.

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## EFFECTS OF DIETARY DEPLETION OF RIBOFLAVIN<sup>1</sup>

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ONE FIGURE

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Stannus ('13) described many of the clinical symptoms of ariboflavinosis more than 20 years before riboflavin was discovered. Subsequent reports produced additional evidence of the dietary nature of these symptoms (Bahr, '14; Scott, '18). The specificity of some of the clinical signs received scientific confirmation during the period when riboflavin was being concentrated and isolated (Goldberger and Tanner, '25; Moore, '30; Landor and Pallister, '35). The availability of riboflavin made possible the human dietary experiments of Sebrell and Butler ('38), in which lesions attributable to riboflavin deficiency were produced by a regimen containing 0.5 mg of riboflavin per day. Since then much work has been done by many able investigators, but there remains considerable controversy as to what is, or is not, riboflavin deficiency.

Restricting this discussion to attempts to produce signs of pure riboflavin deficiency by means of dietary experiments of

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at least 5 months' duration, it should be noted that recent efforts to produce such an uncomplicated syndrome have not yielded positive results. Williams, Mason, Cusick and Wilder ('43) fed a diet which provided between 0.8 and 0.9 mg (0.35 mg per 1,000 cal.) of riboflavin per day for over 9 months, but no clinical evidence of riboflavin deficiency was produced in that time. Keys, Henschel, Mickelsen, Brozek and Crawford ('44) observed no signs of deficiency on a diet containing 1.0 mg (0.3 mg per 1,000 cal.) of riboflavin per day, fed for 5 months. Horwitt, Liebert, Kreisler, and Wittman ('48) fed a diet which contained between 0.8 and 0.9 mg of riboflavin per day for over two years, and only one subject out of the 22 observed during this period showed any signs that might be attributed to lack of riboflavin. Although in all of these studies the urinary excretion of riboflavin was reduced to minimum levels, the dermatological signs reported by Sebrell and Butler ('38) were not positively reproduced.

Can a pure riboflavin deficiency be produced experimentally? If skin lesions are observed during subsistence on diets low in riboflavin, are these lesions specific signs of riboflavin deficiency? It was decided to try to answer these questions by feeding for over a year a diet containing between 0.5 and 0.6 mg of riboflavin per day. The choice of this level was influenced by the positive results reported by Sebrell and Butler ('38) on 0.5 mg per day and the negative results of more recent workers who fed larger amounts.

#### METHODS OF STUDY

The techniques employed were strongly influenced by the experiences reported by Williams, Mason, Cusick and Wilder ('43). *The selection of subjects* was made in a manner quite similar to that reported by Horwitt, Liebert, Kreisler and Wittman ('48). The subjects for the study were chosen after evaluating the qualities of several hundred mental patients. Choice was based upon chronicity of mental illness, excellence of physical condition, and the presence of a reasonable amount of emotional stability. The psychiatric diagnoses



varied widely, which factor minimized the very unlikely possibility that a metabolic disorder concerned with riboflavin could be related to the mental disorder of the subjects. Choice of subjects was also influenced by attitudes, cooperativeness, physical health and habits of eating. After a three-month trial feeding period on a diet which was made up to resemble that which was to be used later but which provided about twice as much riboflavin (1.1 mg per day), 39 subjects were selected. They were divided into three groups in May, 1947. Group A, 15 subjects, received a diet supplying 0.55 mg of riboflavin per day; group B, 14 subjects, received the same diet but in addition were given a tablet containing 2 mg of riboflavin per day; group C, 10 subjects, ate the regular hospital diet ad libitum. Only one subject (B6) failed to cooperate in eating and he was in the supplemented group. Another subject (B2) was not available for further observation after 8 months of supplementation.

The tentative original plan called for experimental periods of at least one year's duration before riboflavin would be added to the diet of the depleted group. However, the appearance in three subjects of symptoms of dermal lesions necessitated supplementing the diet of these three during the 9th and 10th months of the period of depletion.

#### *Diet and dietary supplements*

The details of the diets and the extensive dietary controls are described elsewhere (Horwitt, Sampson, Hills and Steinberg, '49). In brief, three separate lists of foods, which permitted the serving of menus that were repeated every 9 days, were fed by a kitchen staff of 4 full-time workers in a building especially remodeled for nutritional investigations. The basic diet provided 2,200 cal., 57 gm protein, 65 gm fat and 0.55 mg of riboflavin per day. The natural foods in the diet provided less than 45 gm of protein per day, and it was necessary to add from 10 to 15 gm of calcium caseinate<sup>3</sup> to the

<sup>3</sup> Supplied by the Borden Company.

daily menus in order to avoid the theoretical possibility of an amino acid deficiency.

With one day's standard food ration regarded as 100%, the amount of food was varied with the subject's size and appetite, so that 6 subjects in group A received more or less than 100%. These were subjects A1, A4, A5, A7, A9 and A15, who received 110, 110, 80, 110, 120 and 120%, respectively. All foods were repeated every three days, but the form in which the food was served was varied in a 9-day cycle.

The diet containing approximately 1.1 mg of riboflavin per day which was fed during the three-month preliminary period was similar to the one used in the later experimental period except that the allotment of meat was slightly larger and a flour of higher riboflavin content was used to make the bread.

The following daily supplement of vitamin<sup>4</sup> was given to all subjects on the controlled diets: thiamine hydrochloride 0.6 mg, niacinamide 10 mg, pyridoxine 1 mg, calcium pantothenate 3 mg, biotin 0.05 mg, folic acid 0.1 mg, ascorbic acid 50 mg, vitamin A 4,000 U.S.P. units, and vitamin D 400 U.S.P. units. In addition, 500 mg of dicalcium phosphate were given daily and 180 mg of ferrous sulphate three times weekly. Calculation of the intake of riboflavin on the 0.55 mg diet of the A group on the basis of body weight gives a figure of approximately 9.0  $\mu$ g per kilogram of body weight per day. Analytical control of the riboflavin content of the diet was continuous during the first year of the study. About one-half of all the meals were sampled and assayed by both fluorimetric (Slater and Morell, '46) and microbiological methods (Roberts and Snell, '46).

### *Tests employed*

Since an earlier study (Horwitt and Kreisler, '49) using a diet containing 0.8 to 0.9 mg of riboflavin and 0.4 mg of thiamine per day had produced definite changes in carbohy-

<sup>4</sup>Special formula supplied by Hoffmann-LaRoche, Inc.

hydrate metabolism, it was considered important to repeat some of this work in the present study, in which the allowance of riboflavin was decreased to 0.55 and that of thiamine raised to approximately 0.9 mg per day. The levels in the blood of *glucose, lactic acid and pyruvic acid* were determined before and after oral glucose administration and after a mild exercise test, which was given 60 min. after the ingestion of the glucose. This test, the methods used in the analyses and the use of the results to obtain an index of carbohydrate metabolism have been described by Horwitt and Kreisler ('49).

The 24-hour *urinary excretion* of riboflavin and the excretion of riboflavin during the 4 hours following the injection of 1 mg of riboflavin are described by Horwitt et al. ('49). Blood riboflavin was estimated according to a microbiological method similar to that of Strong, Feeney, Moore and Parsons ('41).

Hematological analyses included determinations of hemoglobin, hematocrit, red blood cell and differential counts. Basal metabolic rates, electrocardiograms, electroencephalograms and orthodiagrams of heart size were obtained before, during and after the periods of dietary depletion. Cephalin-cholesterol flocculation tests, icteric indices and the blood levels of cholesterol, N.P.N., creatinine and phospholipids were also determined.

The large number of *clinical examinations* which were conducted daily, or as often as was necessary, will be described in detail in a paper dealing with this aspect of the experiments. They included neurological tests of reflexes, vibration sense and auditory acuity; tests of visual acuity, flicker fusion, tear flow, dark adaptation, corneal sensitivity and internal ocular pressure; and slit-lamp examination for corneal vascularization. A photographic record was kept of the gait, face, tongue, lips, genitals and finger capillaries. The ability of the subjects to perceive flicker was determined by using a mercury bulb which was illuminated and extinguished at different controlled rates. The point at which the light interruptions were just recognized by the subject was accepted

as the threshold of flicker fusion. Pupil size and pupil contraction time were also determined photographically. Psychiatric evaluations and the psychological testing of behavior were a part of the routine; these will be reported elsewhere.

### *Growth and wound healing of rats*

As an additional test of the riboflavin content of the basal diet, homogenized aliquots (with additional iron provided by adding 0.17 gm ferric ammonium sulphate to 100% of the diet) were fed to rats. In a typical experiment 22 weanling rats were divided into two groups. Half of these animals received the basal diet fed to the human subjects in group A and the other half the same diet with 60  $\mu$ g of riboflavin added per day per rat. Simultaneously, in order to facilitate the availability of animals with marked deficiencies of riboflavin, a diet containing half as much riboflavin as that provided for the subjects in group A was fed to another group of 18 rats. The latter diet differed from the A group diet in that all meat and bread were removed and an equivalent amount of protein in the form of lactalbumin<sup>5</sup> added. This diet provided approximately 300  $\mu$ g of riboflavin for each 100% of the diet, or 0.5  $\mu$ g per gram of dry weight. Half of the animals on this diet received 60  $\mu$ g of riboflavin daily as a supplement.

While attempting to evaluate the effect of riboflavin on the dermal lesions observed in humans, preliminary studies of wound healing were made on 6 150-day-old rats, 4 of which were on the deficient diet and two of which received supplementary riboflavin daily. Circular wounds 1 cm in diameter were made on the dorsal surfaces of the rats by removing the skin to the depth of its normal separation from underlying tissue, according to the technique described by Paul, Paul, Taylor and Marsters ('45). The rate and character of wound healing were observed and compared with the amount of riboflavin in the diets of the animals.

<sup>5</sup> Supplied by the Borden Company.

## OBSERVATIONS

*Blood glucose, lactic acid and pyruvic acid*

These data for the subjects in groups A and B are given in table 1. The indices of carbohydrate metabolism (CMI) were calculated on the basis of the analysis of 66-minute samples, obtained 5 minutes after 1 minute of stair climbing, done 60 minutes after the oral administration of glucose. Unlike the results of the previously reported study of thiamine deficiency (Horwitt and Kreisler, '49), there were no apparent changes in the carbohydrate metabolism; i.e., no accumulation of lactic and pyruvic acids in the blood. The data of the completed group compare favorably with those previously published and obtained with subjects who had received diets which were adequate in all respects.

*Urinary excretion of riboflavin*

These details are reported elsewhere by Horwitt et al. ('49). In brief, the average subject dropped from an original excretion of 400  $\mu$ g in 24 hours, on the hospital diet which contained 1.6 mg per day, to an average of 112  $\mu$ g on the 1.1 mg diet used in the preliminary period. Seven days after the 0.55 mg diet was started, the average excretion of the subjects of group A dropped to 50  $\mu$ g; it persisted at that level for 9 months and then fell to as little as 36  $\mu$ g per day. Excretions after supplementation with riboflavin depended upon the size of the supplement. Thus, when 2 mg were given once, twice or three times a day, tissue saturation as determined by the 4-hour excretion test was obtained at 38, 25 and 11 days, respectively.

The many analyses of the constituents of the blood did not reveal any differences that could be interpreted as being related to deficiency of riboflavin. The hematological examinations and liver function tests all gave results which are considered to be within "normal" ranges. No evidence of pathological findings was noted in examination of the electrocardiograms, heart orthodiagrams or basal metabolic rates.

TABLE 1  
Average glucose, lactate and pyruvate after glucose administration and exercise (milligram per 100 ml of blood)

TEST	TIME OF TEST					
	April '47	July '47	Sept. '47	Nov. '47	Mar. '48	Aug. '48
Basal	Glucose	102 ± 7	102 ± 10	103 ± 10	106 ± 7	98 ± 7
	Lactate	8.0 ± 2.1	9.4 ± 2.5	8.6 ± 2.1	7.7 ± 1.6	7.5 ± 1.8
	Pyruvate	0.95 ± 0.18	0.94 ± 0.24	0.85 ± 0.22	0.85 ± 0.14	0.85 ± 0.16
60 minutes	Glucose	155 ± 23	142 ± 16	157 ± 30	163 ± 32	144 ± 28
	Lactate	13.5 ± 3.2	12.5 ± 2.3	14.2 ± 2.2	13.9 ± 2.8	12.1 ± 4.1
	Pyruvate	1.38 ± 0.22	1.24 ± 0.24	1.43 ± 0.26	1.32 ± 0.28	1.29 ± 0.13
66 minutes <sup>1</sup>	Glucose	140 ± 18	133 ± 22	145 ± 28	144 ± 26	148 ± 51
	Lactate	18.7 ± 2.8	18.8 ± 3.2	21.0 ± 5.0	18.8 ± 3.3	18.6 ± 6.5
	Pyruvate	1.63 ± 0.23	1.57 ± 0.26	1.56 ± 0.26	1.45 ± 0.22	1.51 ± 0.22
CMI <sup>2</sup>		7.6 ± 3.1	8.0 ± 4.6	7.7 ± 5.1	6.1 ± 3.5	7.0 ± 4.0
120 minutes	Glucose	121 ± 23	120 ± 22	132 ± 22	131 ± 18	123 ± 15
	Lactate	14.3 ± 3.4	14.4 ± 3.8	13.2 ± 2.9	13.1 ± 1.5	13.3 ± 2.5
	Pyruvate	1.23 ± 0.17	1.24 ± 0.25	1.23 ± 0.28	1.16 ± 0.27	1.15 ± 0.22
Basal	Glucose	102 ± 8	96 ± 5	104 ± 9	106 ± 4	98 ± 4
	Lactate	7.1 ± 1.6	9.1 ± 2.2	8.3 ± 1.6	7.3 ± 1.7	8.2 ± 1.6
	Pyruvate	0.91 ± 0.22	0.92 ± 0.21	0.77 ± 0.17	0.97 ± 0.20	0.86 ± 0.16
60 minutes	Glucose	142 ± 30	141 ± 28	144 ± 22	156 ± 25	155 ± 23
	Lactate	13.5 ± 3.0	13.6 ± 3.0	14.6 ± 3.0	13.0 ± 2.7	13.7 ± 4.3
	Pyruvate	1.33 ± 0.25	1.33 ± 0.28	1.32 ± 0.28	1.32 ± 0.26	1.35 ± 0.21
66 minutes <sup>1</sup>	Glucose	137 ± 26	134 ± 23	135 ± 21	144 ± 28	140 ± 20
	Lactate	19.3 ± 4.2	18.3 ± 4.1	19.6 ± 4.4	18.3 ± 2.4	20.5 ± 4.0
	Pyruvate	1.66 ± 0.28	1.48 ± 0.29	1.50 ± 0.25	1.66 ± 0.22	1.67 ± 0.41
CMI <sup>2</sup>		7.9 ± 2.4	6.8 ± 3.3	7.6 ± 2.8	7.2 ± 2.9	8.8 ± 3.5
120 minutes	Glucose	131 ± 29	119 ± 26	130 ± 22	126 ± 17	124 ± 19
	Lactate	12.8 ± 2.8	12.6 ± 3.5	11.2 ± 1.9	12.8 ± 2.7	11.5 ± 1.9
	Pyruvate	1.24 ± 0.18	1.18 ± 0.37	1.12 ± 0.28	1.12 ± 0.20	1.09 ± 0.21

<sup>1</sup> Immediately after the 60-minute blood sample is drawn, the subject walks up and down a flight of stairs for one minute. Five minutes later a blood sample is drawn, and the latter is referred to as the 66-minute sample.

<sup>2</sup> The glucose, lactic and pyruvic acid data observed from the 66-minute sample are calculated according to the formula:

$$CMI = \frac{L - \frac{G}{10} + 15P - \frac{G}{10}}{2}, \text{ where G, L and P are glucose, lactic acid and pyruvic acids in milligrams per cent, respectively.}$$

Toward the end of the deficiency period psychological tests of mental fatigue produced results which indicated that the subjects in group A were less efficient than the subjects in the supplemented group. These tests will be repeated after group A has been supplemented with riboflavin for one year, at which time they will be evaluated.<sup>6</sup>

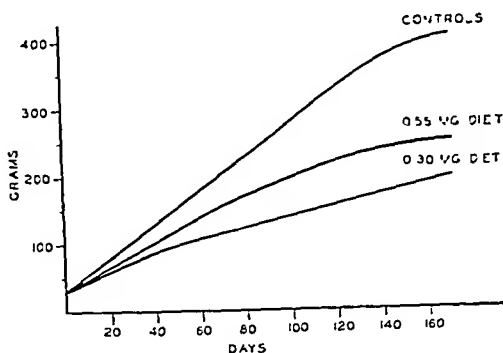


Fig. 1 The growth of rats on the human experimental diets. The 0.55 mg diet was the homogenized equivalent of the food fed to group A subjects; the 0.30 mg diet was similar to the latter except that meat and bread were removed and an equivalent amount of protein added as lactalbumin. The controls received the same diets but 60  $\mu$ g of riboflavin were added daily. The controls for the two diets are plotted together because the results obtained when riboflavin was added were very similar.

### *Experiments with rats*

That the group A diet was inadequate with respect to riboflavin for the optimum growth of rats is shown in figure 1. Good growth was obtained when riboflavin was added to the diet.

Of possible significance is the observation, common to most nutritional deficiency studies, that though the deficient animals had more skin lesions around the head, such lesions were not completely absent in the control group. However, in the latter group any such lesion would heal soon after appearing, whereas a similar one would tend to persist in the animal on the deficient diet.

<sup>6</sup> These psychological observations will be reported by Miss Thelma Johnson.

The healing of the circular wounds produced in the rats was directly related to the amount of riboflavin in their diets. There was a marked difference in the rates of tissue proliferation in the rats fed different levels of riboflavin. The wounds of the animals who received added riboflavin developed a scab the first day and decreased in size in three days. At 14 days only a tiny crust was left, which was lost at 20 days. No sign of previous injury was detectable at 28 days, at which time there was complete regrowth of hair. The wounds of animals on the A group diet healed more slowly. There was some wound contraction on the 4th day but the area was quite moist. On the 28th day healing was far from complete, and after 54 days the wounds were still evident, with marked retardation of the fur regrowth. Bosse and Axelrod ('48) made similar observations with respect to rat diets much more deficient in riboflavin than those we fed. The present preliminary rat study with diets of borderline deficiency will be repeated, using larger numbers of animals and paired-feeding techniques.

#### *Clinical examinations*

Cheilosis,<sup>7</sup> involving the vermilion border of the lips and including vertical fissuring, crusting and desquamation, was seen in only one subject (A15). In his case the lesion was never more than minimum, and was manifested only by redness and slight vertical fissuring in a lip that was naturally almost abnormally protruberant. Angular stomatitis, the crusting and fissuring of the oral angles, appeared for varying periods of time in three members (A1, A5 and A11) of the depleted group but in none of the supplemented subjects. In none of these three was there evidence of excessive salivation, or of inefficient dentures. In the case of subject A1, a bacteriological investigation of the wound produced no evidence of a mycotic infection.

The first manifestation of possible deficiency of riboflavin appeared in subject A1 in September 1947, 4 months after

<sup>7</sup>Dr. Cleveland J. White and Dr. H. S. Steinberg gave dermatological advice and treatment during the period of observation of the skin lesions.



the beginning of the 0.55 mg diet. This consisted of a lesion, which was diagnosed as typical herpes simplex vesicle, at the left oral angle. During the succeeding 6 weeks a definite horizontal fissure with crusting appeared at both oral angles. Local therapy with zinc oxide and camphorated oil assisted healing of the right oral angle in less than three weeks. That on the left, however, remained in a fluctuating state of remission and exacerbation and did not heal completely until after riboflavin was administered. In addition to the angular stomatitis, subject A1 had a scaling, crusting lesion of the scalp which was resistant to routine therapy with sal-sulphur ointment. A similar condition was present in the hairy areas of the chest. On the face, severe vertical fissuring appeared at the naso-labial folds and eroded through the muco-cutaneous border into the nostrils. Small fissures also appeared at the orifice of the nares. All of these conditions healed very rapidly after the administration of riboflavin.

The second subject (A5) developed similar lesions without the appearance of any pre-existing herpes. They were noted in March 1948, two weeks after the appearance of a severe scrotal dermatitis (see below). Because of the severity of the latter lesion, the patient was given 6 mg of riboflavin per day. This supplementation was begun 5 days after the appearance of small fissures at both oral angles. Marked improvement was observed within 48 hours and the lesions healed completely in two weeks. It may be significant that this subject was the only poor eater in group A. He maintained his weight of 67 kg on an 80% diet, and so received only 0.44 mg of riboflavin per day.

In the third subject (A11) to develop an angular fissure, it healed without therapy. The lesion had been present constantly in varying degrees for 5 weeks. There was no recurrence.

#### *Scrotal skin changes*

The appearance of redness, scaling and desquamations of the superficial layer of the skin of the scrotum was first

observed in subject A8 in the 8th month of the 0.55 mg diet. The skin of the anterior surface of the scrotum was markedly reddened. The lesion was discrete, bilateral, and not connected in mid-line. A similar lesion appeared in 12 of the 15 subjects in group A between the 8th and 10th month after the beginning of the 0.55 mg diet. It was most severe in subject A5, appearing markedly reddened, raw and weeping, and extending to the adjacent areas of the thighs. Elevation of the scrotum and warm saline applications ameliorated the acute symptoms but marked erythema persisted, followed by induration of the skin. Three weeks after the onset of this condition the administration of riboflavin produced striking improvement within 48 hours, and all signs of inflammation, except for residual erythema, disappeared within a week. The marked scrotal lesion of subject A8 responded equally dramatically within one week after the administration of riboflavin. This lesion had been present for 6 weeks with little change before riboflavin was administered.

The remaining 10 subjects showed various degrees of the lesions described, i.e., moderately severe to mild scrotal erythema and scaling. Only a few of them complained of itching.

After all of the lesions had appeared in the A group, three subjects in the B group developed a scrotal erythema with little or no scaling. This prompted an investigation of several hundred other hospital patients, who presumably were adequately nourished. Scrotal erythema, but no scaling, was found in a large percentage of those who were incontinent. The basic irritation of the skin of the subjects deficient in riboflavin was probably explained by poor hygiene. However, no bacterial or mycotic infection could be found. In the absence of adequate riboflavin, tissue repair became retarded and thereby the development of the more severe manifestations of scrotal dermatitis was promoted. The possibility that cleaning agents used in the hospital laundry may have been a contributing factor was considered but not established.

*Flicker fusion*

The threshold for flicker fusion, when tested at a distance of 6 feet from the source of flicker, showed an increase in three subjects in group A: A4, A6 and A12. None of the subjects in group B showed similar changes. When in September 1948, the testing was done at a distance of 12 feet from the source of flicker, all but one of the A group showed a marked increase as compared to the responses of the subjects in group B.

## DISCUSSION

The report of Sebrell and Butler ('38) that signs of deficiency of riboflavin can be obtained at a riboflavin level of 0.5 mg per day has been confirmed by the above observations, in which angular stomatitis was obtained at a level of 0.6 mg or less per day. The scrotal dermatitis we observed may be similar to that reported on the severe vitamin B complex deficiency diet used by Berryman et al. ('47). Whether or not these and the other abnormalities observed are specific signs of riboflavin deficiency is not certain. That riboflavin is necessary for the growth of tissue is generally agreed. Any local trauma to the skin must be repaired by new growth. Will not the signs of deficiency, then, be partially dependent upon the need for riboflavin for local tissue replacement? If this is so, then the location of disorders of the skin will depend upon the presence of local irritations, infections and other traumas as primary causes. Thus a slight sore, originally diagnosed as a common "herpes simplex," became a typical angular stomatitis; an itchy scalp became a seborrheic dermatitis; and a sore nose which appeared in conjunction with a mild upper respiratory infection became inflamed, the inflammation spreading over the anterior portion of the septum and a fissure appearing at the naso-labial fold. All these lesions were improved spectacularly when riboflavin was administered after other therapy had failed. The same can be said of the scrotal dermatitis which was so prevalent in the subjects of group A, although the primary cause of this

particular lesion remains unknown. It may be that many of the "classical" signs of riboflavin deficiency are subject to controversy because the different patients observed had different initial traumata or none at all.

Consider the rats used in the present experiments. The control animals had occasional sores which came and went, but the sores which appeared in the deficient animals persisted. This was true for all wounds, including those which appeared for unknown reasons.

The urinary excretions of riboflavin correlated well with the amounts of riboflavin ingested. About 25% of the amount of riboflavin in a diet containing 1.6 mg per day is excreted. Since the 24-hour excretion drops markedly to approximately 10% when the intake of riboflavin is lowered to 1.1 mg per day, and remains at 10% for lower levels of intake, it can be suggested that the *basal* requirement for an adult is between 1.1 and 1.6 mg per day (Horwitt et al., '49). Emergency requirements during illness and for repair of tissue would necessarily be greater.

Three of the subjects, A1, A5 and A8, who had the most severe lesions had auburn beards. This may or may not have been a coincidence, but it is thought-provoking to add to this fact the observation that the one subject (BY3) in the previous project in this laboratory (Horwitt et al., '48, p. 55) who developed angular stomatitis is a distinct "red-head." For almost a year this man had been on a diet which provided 0.75 mg of riboflavin per day. The significance of the rather severe left oral lesion which spread over the left cheek was minimized because of a history of minor oral lesions which developed when the subject had been receiving riboflavin as a dietary supplement. This is another demonstration that such oral lesions per se are not necessarily due to riboflavin deficiency; but when they become worse and do not heal readily until after riboflavin is provided, then ariboflavinosis is a proper diagnosis.

The "yellow enzymes" are necessary in such a large number of cellular reactions (Sumner and Somers, '43) that it

is surprising that many tests of efficiency in addition to the flicker fusion test and psychological tests of mental fatigue have not produced positive results. The inability to find any changes in the metabolites in the body fluids may be because the cell, once matured, requires only a minimum amount of riboflavin. However, the rate of replacement of such cells with others may be dependent upon a more abundant supply of riboflavin, obtainable either from the diet or through the breakdown of less vital tissue.

#### SUMMARY

The effects of diets restricted in riboflavin but otherwise adequate were observed in 15 male subjects. Fourteen other subjects were observed simultaneously as controls. Depletion with respect to riboflavin on a diet of 0.55 mg in 2,200 cal. was maintained for from 9 to 17 months, during which period angular stomatitis, seborrheic dermatitis, scrotal skin lesions, and diminution of ability to perceive flicker were observed.

Simultaneous experiments in which the same diet was fed to rats confirmed the inadequacy of the diet. In these animals retardation of wound healing was conspicuous.

Studies of the excretion of riboflavin in the urine suggest that the riboflavin requirement of a resting adult is between 1.1 and 1.6 mg per day. A reserve of riboflavin cannot be maintained on levels of intake below 1.1 mg.

Since previous studies revealed few abnormalities when the intake of riboflavin was above 0.6 mg per day, it may be concluded that allowances below this amount are insufficient to support normal tissue repair. The type of abnormality then encountered will be dependent upon the trauma, irritation, infection or other injuries to which the tissues are subjected.

Changes in the blood levels of lactic and pyruvic acids were not obtained on diets low in riboflavin, as opposed to the marked changes in carbohydrate metabolism observed during thiamine deficiency.

## ACKNOWLEDGMENTS

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# THE RELATIONSHIP OF CARBOHYDRATE METABOLISM TO PROTEIN METABOLISM

## III. FURTHER OBSERVATIONS ON TIME OF CARBOHYDRATE INGESTION AS A FACTOR IN PROTEIN UTILIZATION BY THE ADULT RAT

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### ONE FIGURE

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When extra carbohydrate or extra fat is superimposed on an adequate diet, N is retained by the body (Cuthbertson and Munro, '37). Larson and Chaikoff ('37) made the interesting observation that time of ingestion is a factor in the protein-sparing action of extra carbohydrate. In their experiments, dogs were brought into N equilibrium on a mixed diet fed once daily and were then given single feedings of additional carbohydrate. It was found that this extra carbohydrate induced N retention only when given within a 4-hour interval before or after the mixed meal. From these experiments it was concluded that proximity of the additional carbohydrate to the protein of the mixed meal was essential for the protein-sparing effect to take place.

In 1939, Cuthbertson and Munro extended the question of time relationships to the protein and carbohydrate contained in ordinary diets. Experiments were carried out with adequate

<sup>1</sup> This work was carried out during the tenure of a Rockefeller Fellowship administered by the British Medical Research Council. Present address: Biochemistry Department, The University, Glasgow, Scotland.

diets which could be separated into two portions, one containing all or almost all of the carbohydrate and some of the fat of the diet, the other containing all of the protein and the remainder of the dietary fat. Four human subjects were studied and each received a constant daily amount of one diet throughout the experiment. When the carbohydrate-fat and protein-fat portions of the diet were eaten separately, the urinary N output was about 2 gm greater than when the meals consisted of a mixture of these portions. An increased output of urinary N was also observed when a diet given to adult rats was separated into carbohydrate-fat and protein-fat meals (Cuthbertson, McCutcheon and Munro, '40). The adverse effect on protein metabolism produced by eating the carbohydrate-fat and protein-fat portions of a diet at different times was attributed to separation of the dietary protein and carbohydrate, and it was concluded that time of carbohydrate ingestion plays an important part in protein utilization under normal nutritional circumstances. This hypothesis seems of sufficient interest to justify more rigorous examination, with a view to finding out whether changes in time of carbohydrate ingestion can by themselves account for the alterations in N excretion observed in the separation experiments, or whether the simultaneous separation of some of the fat of the diet from the dietary protein plays a part. Further experiments have accordingly been carried out on adult rats, using an experimental design in which the ingestion of only one dietary factor was varied at a time. These experiments show that a change in the time of carbohydrate ingestion can by itself cause alterations in N output. The effect is rather transitory. Changing the time of fat ingestion does not result in any significant alteration in N balance. The evidence for these conclusions follows.

#### EXPERIMENTAL

Adult male rats of the Sprague-Dawley strain were used. The diets consisted of purified ingredients — vitamin-free

casein,<sup>2</sup> corn starch, glucose,<sup>3</sup> lard or corn oil,<sup>4</sup> vitamins, minerals and roughage (wood flock) — and provided daily about 400 mg N and some 1,000 to 1,200 cal. of metabolizable energy per square meter of body surface area (the latter was estimated using the formula of Lee and Clark, '29). The animals drank only distilled water.

The rats were fed twice daily, at 9 A.M. and at 5 P.M. The food was weighed with great care into non-spilling feeding dishes and was moistened with water. The morning meal always contained the vitamins, minerals and wood flock of the diet in the form of a supplement<sup>5</sup> which also provided 0.83 gm carbohydrate, 0.28 gm fat and 3 mg N. The evening feed contained all the protein (casein) of the diet. Preliminary experiments showed that casein fed alone is unpalatable but is eagerly consumed if made into a paste with  $\text{NaHCO}_3$ . Throughout these experiments the casein was therefore mixed with 0.15 gm  $\text{NaHCO}_3$ , whether or not there were other constituents in the meal. The carbohydrate and fat (except for the supplement mentioned above) were mobile constituents of the diet, being given with either the morning or the evening feeding, according to the requirements of the experiment. The feeding routine of a typical experiment (the first to be described) is shown in table 1. The total constituents of the diet were kept constant throughout the experiment, the only variable factor being time of carbohydrate ingestion. During the first period all of the dietary carbohydrate and fat was given in the morning feeding. In the second period, carbohydrate was transferred from

<sup>2</sup> Labeo.

<sup>3</sup> Cerelease.

<sup>4</sup> Mazola.

<sup>5</sup> Two grams of the supplement contained 829 mg starch, 226 mg wood flock, 226 mg cod liver oil, 54 mg wheat germ oil, 169 mg sodium chloride, 167 mg dicalcium phosphate, 112 mg potassium citrate, 77 mg calcium carbonate, 36 mg dipotassium phosphate, 19 mg magnesium carbonate, 7.5 mg ferric citrate, 590  $\mu\text{g}$  manganous sulfate, 80  $\mu\text{g}$  copper sulfate, 40  $\mu\text{g}$  potassium aluminum sulfate, 40  $\mu\text{g}$  cobalt chloride, 20  $\mu\text{g}$  potassium iodide, 20  $\mu\text{g}$  zinc carbonate, 0.4  $\mu\text{g}$  sodium fluoride, 72.4 mg Merck's dry mix (25% choline chloride), 1.8 mg inositol, 900  $\mu\text{g}$  *p*-aminobenzoic acid, 360  $\mu\text{g}$  calcium pantothenate, 180  $\mu\text{g}$  nicotinic acid, 45  $\mu\text{g}$  thiamine hydrochloride, 45  $\mu\text{g}$  riboflavin and 45  $\mu\text{g}$  pyridoxine hydrochloride.

the morning to the evening feeding. During the third period (not included in table 1), the carbohydrate was transferred back to the morning meal, as in period I. This experiment permits one to compare N balance when protein and carbohydrate are taken separately (periods I and III) with N balance when they are eaten together (period II). In other experiments of similar design, fat was the mobile constituent of the diet.

It was clearly important in experiments involving the separate eating of two dietary constituents that a reasonable interval should elapse between the completion of one meal and

TABLE 1

*Diet used in experiment 1 to study the effect of feeding carbohydrate separately from protein (period I) and together with protein (period II)*

	PERIOD I		PERIOD II	
	Morning feeding	Evening feeding	Morning feeding	Evening feeding
Vitamin-mineral-roughage supplement	2 gm	.	2 gm	..
Corn oil	2.5 ml	.	2.5 ml	..
Glucose	4 gm	.	..	4 gm
Casein		2.5 gm	..	2.5 gm
NaHCO <sub>3</sub>		0.15 gm	..	0.15 gm

the start of another meal. To ensure this, the feeding dishes were inspected at intervals throughout the day and if, as happened on rare occasions, a rat could not be coaxed to clean up the morning feeding several hours before the evening meal, the residue was removed and given with the morning feeding on the following day. Rats consistently refusing food were taken off experiment. There was never any difficulty in persuading the rats to clean up the evening meal quickly, once it had been appreciated that casein fed alone must be partly brought into solution with mild alkali in order to make it palatable.

Throughout the experiments the rats were housed in metabolism cages of the type used in this laboratory in the deter-

mination of the biological value of proteins. During the first 7 days of each experiment the rats were fed the full amount of the diet but the urine and feces were discarded. After this preliminary period of adjustment to the diet, urine and feces were collected separately at 9 A.M. every day; urine was stored with the addition of strong HCl. In most experiments analyses were made of the total urinary and fecal collections of 4-day periods. Two experiments were exceptions: in experiment 1 daily urine collections were analyzed, and in experiment 7 estimations were made on urine and feces collected over 7-day periods. Iron oxide was employed as a fecal marker to indicate the beginning and end of periods.

Food, feces and urine were analyzed for total N by macro-Kjeldahl estimation; mercury was used as the catalyst and digestion of food and feces samples was continued for 4 hours after clearing of the solution. This period of digestion seems to be adequate. One sample of casein analyzed in this way was found to contain 15.64% of N on a moisture-, ash- and fat-free basis; this agrees reasonably well with the average figure of 15.73% obtained for casein by Chibnall, Rees and Williams ('43) after a critical investigation of different catalysts and periods of digestion. Urine samples were digested for one hour after clearing. The data have been treated statistically and probabilities of less than 0.05 have been considered as likely to be significant, while values less than 0.01 have been regarded as highly significant.

## RESULTS

### 1. *Is protein utilization affected by the presence of carbohydrate at the same meal?*

*Experiment 1.* Four rats with an average initial weight of 373 gm were used. They received a diet containing 2.5 gm protein,, 2.5 gm fat and 4.8 gm carbohydrate, 4 gm of carbohydrate (glucose) being mobile. The changes in diet during the course of the experiment are shown in table 1, and the mean daily output of N in the urine is recorded in figure 1.

During the preliminary week of adjustment to the diet and for the first few days of urinary collection (period I), all of the carbohydrate of the diet was given in the morning feeding and was therefore eaten separately from the protein of the diet. The output of urinary N remained reasonably constant during this period. Then the 4 gm of mobile carbohydrate were

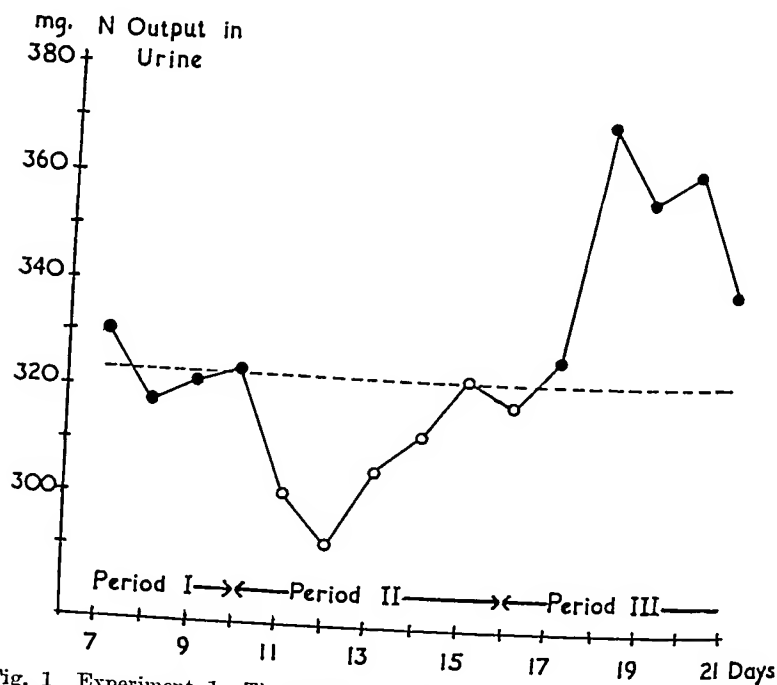


Fig. 1 Experiment 1. The effect of urinary output of altering the time of carbohydrate ingestion. Periods I and III, protein and carbohydrate eaten separately; period II, protein and carbohydrate eaten together.

removed from the morning feeding and given with the casein of the evening feeding (period II). There was at once a depression of N output which was, however, quite transitory, and after 4 days N output had regained levels similar to the average output of period I (shown in figure 1 by the dotted line). Then the mobile carbohydrate was once more given in the morning feeding (period III) and N excretion responded by increasing markedly; this effect was also of short duration,

and within 5 days N output had fallen nearly to the average level of period I.

During the experiment feces were collected and it was possible to compute the N balance of each rat for each period. In period I, when protein and carbohydrate were eaten separately, the mean daily N balance of the 4 rats was + 22.4 mg N; in period II, when protein and carbohydrate were taken at the same meal, it was + 41.5 mg; in period III, when protein and carbohydrate were again separated, the mean daily N balance was - 4.2 mg. Analysis of variance shows that these mean values differ from one another significantly (for the difference between periods I and II,  $P = 0.05-0.02$ ; for the difference between periods I and III,  $P = 0.02-0.01$ ; and for the difference between periods II and III,  $P$  is less than 0.01). A point of some interest is that, taking the mean daily N balance of period I as a base line, the extra N retained during the 6 days of period II amounted to 114.6 mg per rat, while the extra N excreted during the 5 days of period III was 133.0 mg. The fact that these figures are of the same order of magnitude suggests that the N retained when carbohydrate and protein were associated in period II was all eliminated again when the times of carbohydrate and protein ingestion were separated in period III.

*Experiments 2, 3 and 4.* Three confirmatory experiments<sup>6</sup> were carried out along the same lines as experiment 1, except for two points. First, only one dietary change was made, from carbohydrate and protein fed separately to carbohydrate and protein fed together. Secondly, a control group of rats received the diet without any change being made in the order of feeding during the experiment. In experiments 2 and 4 the diet was the same as that used in experiment 1; in experiment 3 the fat was reduced to 2.1 gm and the carbohydrate to 4.3 gm, and only 3.5 gm of glucose were transferred from the morning to the evening meal. Table 2 gives the mean daily N balances computed for 4-day periods beginning after the rats had been

<sup>6</sup> A further experiment along these lines had to be abandoned because the control group showed a significant improvement in N balance during period II.

TABLE 2

Experiments 2, 3 and 4. The effect of transferring carbohydrate from the morning (protein-free) meal to the evening (protein-containing) meal. (Experiment 4 also includes results from a group in which carbohydrate was added to the diet at the evening meal during period II)

EXPT. NO.	GROUP	NO. IN GROUP	MEAN INITIAL WEIGHT	DAILY N INTAKE	PERIOD I		PERIOD II		PERIOD III	
					Evening feeding	Mean daily N balance	Evening feeding	Mean daily N balance	Evening feeding	Mean daily N balance
			gm	mg		mg		mg		mg
2.	Carbohydrate transferred	4	314.5	369.2	Protein	+ 32.8	Protein and carbohydrate	+ 46.4	Protein and carbohydrate	+ 20.7
	Control	5	307.0	369.2	Protein	+ 34.0	Protein	+ 26.4	Protein	+ 27.6
	Carbohydrate transferred	4	311.1	373.3	Protein	+ 25.5	Protein and carbohydrate	+ 57.2	Protein and carbohydrate	+ 36.4
3.	Control	4	307.8	373.3	Protein	+ 29.0	Protein	+ 29.4	Protein	+ 29.5
	Carbohydrate transferred	3	326.5	374.3	Protein	+ 52.9	Protein and carbohydrate	+ 92.6		
	Carbohydrate added	3	328.0	374.3	Protein	+ 40.0	Protein and carbohydrate	+ 90.8		
4.	Control	3	329.5	374.3	Protein	+ 41.8	Protein	+ 34.3		

In experiment 2 the mean daily N balance of the experimental group during period II is significantly more positive than the mean N balance of the control group ( $t = 3.18$ ;  $P = 0.02-0.01$ ).

In experiment 3 the difference in mean daily N balances of the experimental and controls groups during periods II and III combined is significant ( $t = 3.07$ ;  $P = 0.05-0.02$ ).

In experiment 4, comparison of the N balance values obtained during period II after adjustment by co-variance for differences in N balance during period I shows that the values for the first two groups are very significantly different from the mean value for the control group ( $P < 0.01$ ).



on the diet with carbohydrate and protein fed separately for one week. The time at which the mobile carbohydrate was fed during each period is indicated in the table by the composition of the evening meal.

All three experiments demonstrate that N balance improves when carbohydrate is transferred from the protein-free meal to the protein-containing meal without altering the total constituents of the diet. In each experiment the effect is statistically significant by comparison with the behavior of N balance in the corresponding control group. In experiments 2 and 3, carbohydrate and protein were fed together for two successive 4-day periods (periods II and III); in experiment 2 the beneficial effect on N balance was limited to period II and in experiment 3 it was mainly confined to period II. Thus the association of carbohydrate and protein at the same meal does not result in a permanent improvement in N balance. This finding is in agreement with the results of experiment 1.

Experiment 4 includes an additional feature. During period II the effect of transferring carbohydrate from the protein-free to the protein-containing meal was compared with the effect of *adding* the same amount of carbohydrate (4 gm glucose) to the diet along with the protein meal. The added carbohydrate had the same effect on N balance as the transferred carbohydrate, which indicates that the essential condition causing N retention is the association at the same meal of protein and carbohydrate. It should be added that N balance was studied in the second group for a further 8 days and it was found that the addition of supplementary carbohydrate to the evening meal resulted in a more prolonged improvement in N balance than could be achieved by simply transferring carbohydrate from the morning meal.

2. *Is protein utilization affected by the presence of fat in the protein meal?*

*Experiments 5 and 6.* In experiment 5, the effect of transferring fat from the protein-free meal to the protein-containing

meal was compared with the effect of a similar transfer of carbohydrate. In experiment 6 these transfers were carried out in the opposite direction. The diet in each experiment was the same as that used in experiment 1; 2.2 gm corn oil and 4 gm glucose were the mobile constituents.

In experiment 5 the rats were divided into three groups, each of which started by receiving all the dietary constituents except the protein in the morning meal. The mean daily N balances for the first 4-day period of collection (period I) are given in table 3. During the next 4 days of the experiment (period II), the first group were given the mobile fat in the evening meal. The second (control) group received the diet as fed during period I, and for the third group the mobile carbohydrate was transferred to the evening meal. Comparison of the mean N balances during periods I and II shows that the balances were slightly less favorable during period II in the case of the control group and the group receiving fat in the evening meal, but the group receiving carbohydrate with the protein of the evening meal had a significantly better N balance.

In experiment 6, in which the procedure was reversed, N balance was not affected by transferring fat from the evening to the morning meal, whereas N balance was adversely affected by a similar change in the time of carbohydrate ingestion (table 3). This difference in the behavior of the two groups is statistically highly significant.

*Experiment 7.* This comprises a series of three successive studies in which carbohydrate or fat or both were removed from a protein-containing meal. The diet supplied 2.8 gm protein, 2.3 gm fat and 6.5 gm carbohydrate. In each experiment the rats began by receiving 1.5 gm of the dietary fat (as lard) and 3 gm of carbohydrate (equal parts starch and glucose) in the evening meal with the protein of the diet. After the usual preliminary period, urine and feces were collected for 7 days (period I). During the next 7 days (period II) the three groups of rats received the diet arranged in different ways. In the first group, the fat was transferred from the evening meal to the morning meal, leaving the carbohydrate still in association

TABLE 3

Experiment 5. The effect of transferring fat compared with the effect of transferring carbohydrate from the morning (protein-free) to the evening (protein-containing) meal  
 Experiments 6 and 7. The effect of transferring fat or carbohydrate or both from the evening (protein-containing) to the morning (protein-free) meal

EXPT. NO.	GROUP	NO. IN GROUP	MEAN INITIAL WEIGHT gm	DAILY N INTAKE mg	PERIOD I		PERIOD II		DIFFERENCE IN MEAN DAILY N BALANCE	
					Dwelling feeding	Mean daily N balance	Dwelling feeding	Mean daily N balance	11-1	t
5.	Pat transferred	8	317.7	371.7	Protein	+ 25.6	Protein and fat	+ 23.2	mg - 2.4	0.55
	Control	8	315.4	371.7	Protein	+ 36.9	Protein	+ 29.3	mg - 7.6	0.6-0.5
	Carbohydrate transferred	7	319.6	371.7	Protein	+ 41.4	Protein and carbohydrate	+ 66.2	mg + 24.8	3.91 < 0.01
6.	Pat transferred	3	336.3	374.3	Protein and fat	+ 29.9	Protein	+ 36.4	mg + 6.5	6.44 < 0.01
	Carbohydrate transferred	3	338.8	374.3	Protein and carbohydrate hydrate	+ 39.2	Protein	+ 9.7	mg - 29.5	
7.	Pat transferred	4	463.5	409.8	Protein, fat and carbohydrate hydrate	+ 39.5	Protein and carbohydrate hydrate	+ 46.5	mg + 7.1	0.6-0.5
	Carbohydrate transferred	4	466.5	412.8	Protein, fat and carbohydrate hydrate	+ 51.1	Protein and fat	+ 16.0	mg - 35.1	8.67 < 0.01
	Carbohydrate and fat transferred	4	446.3	415.7	Protein, fat and carbohydrate hydrate	+ 39.3	Protein	+ 28.3	mg - 10.9	6.42 < 0.01

with the protein. This change did not affect N balance. In the second group, the carbohydrate was transferred to the morning meal, leaving the fat with the protein of the evening meal; N balance was adversely affected by this separation of carbohydrate and protein. In the third group, all of the carbohydrate and fat in the diet was fed in the morning meal, leaving only protein in the evening meal during period II; N balance was again adversely affected. Thus those changes which affected the time of ingestion of carbohydrate caused highly significant alterations in N balance, whereas the one change which affected only the time of fat ingestion did not alter N balance.

*3. Is protein utilization affected by fat given a short time before the protein meal?*

*Experiment 8.* The failure of fat fed with protein to influence protein utilization might conceivably be due to a lack of coincidence in the times of fat and protein metabolism. There is no reason to believe that fat is more rapidly absorbed and metabolized than protein; the only possibility worth considering is whether it is too slowly utilized to spare protein. An experiment was therefore carried out in which fat was given some time before the protein meal and the effect on N balance examined. The diet used contained 2.5 gm protein, 2.1 gm fat and 4.3 gm carbohydrate; 1.8 gm fat (corn oil) and 3.5 gm carbohydrate (glucose) were the mobile constituents. The rats were divided into 4 groups. During the preliminary week of feeding and for the first 4-day period of urinary and fecal collection, all 4 groups received the whole of this diet except the protein in the morning meal. During this first period the different groups had mean daily N balances of similar magnitude (table 4). During the next 4-day period (period II) the groups received different dietary treatments. The first group were fed the diet as given in period I, and N balance remained unchanged. The second group of rats were given the mobile fat one and one-quarter hours before the evening (protein-containing) meal; N balance was not different from that of

TABLE 4

*Experiment 8. The effect of removing fat from the morning (protein-free) meal and giving it shortly before the evening (protein-containing) meal*

GROUP	NO. IN GROUP	MEAN INITIAL WEIGHT	DAILY N INTAKE	PERIOD I		Evening feeding	PERIOD II		DIFFERENCE IN MEAN DAILY N BALANCE
				Evening feeding	Mean daily N balance		Evening feeding	Mean daily N balance	
Controls	4	307.8	373.3	Protein	+ 29.0	Protein	Protein	+ 29.4	+ 0.4
Fat given 1½ hrs. before	4	308.0	373.3	Protein	+ 20.4	Fat before protein	Fat before protein	+ 19.0	- 1.4
Fat given 2½ hrs. before	4	307.3	373.3	Protein	+ 19.0	Fat before protein	Fat before protein	+ 17.3	- 1.7
Carbohydrate transferred	4	311.1	373.3	Protein	+ 25.5	Protein and carbohydrate	Protein and carbohydrate	+ 57.2	+ 31.7

period I. The third group received the fat two and one-half hours before the feeding of protein, and here also no improvement in N balance resulted. The rats in both of these groups consumed the mobile fat within 10 minutes of being offered it. The 4th group of rats were given the mobile carbohydrate in the evening meal during period II, and in this group the usual improvement in N balance was observed. Comparison of the mean N balances during period II after adjustment by covariance for differences in N balance during period I shows that the behavior of the 4th group is significantly different from the behavior of the other three groups ( $P = 0.05-0.01$ ).

#### DISCUSSION

In experiments 1 through 4, the rats first received a diet with the protein given apart from the other constituents and then they received the same diet with most of the carbohydrate fed along with the protein. The immediate effect of this change in the time of feeding carbohydrate was a considerable improvement in N balance. This finding confirms the hypothesis proposed in earlier papers (Cuthbertson and Munro, '39; Cuthbertson, McCutcheon and Munro, '40), namely, that protein utilization is affected by the presence of carbohydrate in the same meal.

In our earlier experiments, attempts were made to determine the duration of the disturbance in protein metabolism caused by separating dietary protein and carbohydrate, but they were defeated by loss of appetite. The present experiments have not been complicated by this factor, probably because a more complete vitamin mixture was offered. Experiments 1 through 3 show that the disturbances in N balance produced by feeding carbohydrate together with protein are quite temporary, N output returning to its former levels within a few days of a change being made in the time of feeding the carbohydrate. This does not mean that thereafter carbohydrate fed with protein ceases to have any effect on protein metabolism; if the dietary carbohydrate and protein are separated again, there is an immediate loss of N roughly equivalent

to the amount retained when carbohydrate and protein were first taken together (fig. 1). This observation indicates that the continued feeding of carbohydrate with protein is necessary in order to prevent the retained material from being lost again.

One possible explanation of these phenomena is that they are due to variations in the amount of labile protein in the body, especially labile liver protein. It has been established that the amount of protein in the liver is considerably influenced by protein intake, and that the effect of changing from one dietary protein level to another is nearly complete within a few days (Addis, Poo and Lew, '36; Kosterlitz, '47). The improvement in protein utilization brought about by taking carbohydrate along with protein is presumably equivalent to an increase in protein intake and causes a rapid deposition of labile protein in the liver until an equilibrium between synthesis and catabolism has been reached. The attaining of this equilibrium is indicated by the return of N balance to normal levels. If the carbohydrate is then removed from the protein-containing meal, conditions for protein synthesis become less favorable and labile protein is discharged until a new equilibrium between synthesis and catabolism is attained. While it seems likely that changes in the amount of labile liver protein could account for the N balance phenomena of these experiments, one cannot exclude the possibility that other synthetic aspects of protein metabolism are favorably influenced by taking carbohydrate with protein. Cuthbertson, McCutcheon and Munro ('40) did not observe any appreciable difference in growth rate between rats receiving a diet separated into protein-fat and carbohydrate-fat portions and rats receiving the same diet in mixed form. Geiger ('48) has pointed out, however, that the growth rate in these experiments may have been limited by factors other than protein intake. Using a diet which supported better growth, he has been able to demonstrate a retardation of growth rate when protein and carbohydrate were separated by division of the diet into two portions. It would be desirable to study the question of growth

further, using an experimental design similar to the one described in the present paper, in which time of carbohydrate ingestion is the only variable.

Experiments 5 through 7 show quite clearly that no significant change in N balance occurs when the time of fat ingestion is altered to coincide with or differ from the time of taking protein. The possibility that the fat is too slowly metabolized to spare protein taken at the same meal is excluded by experiment 8, in which fat was taken one and one-quarter and two and one-half hours before the protein meal, without affecting N balance. Each of these experiments included a group of rats for which a corresponding change in the time of carbohydrate ingestion was made, and these animals showed significant alterations in N balance. Furthermore, the calories provided by the mobile fat were greater than those provided by the mobile carbohydrate in each of the experiments. We can therefore conclude that fatty acids do not act like carbohydrates in sparing protein given at the same meal. It is, of course, possible that the glycerol of the fat may exert the same effect as carbohydrate, but the amount present in the mobile fat of these experiments was too small to make such an effect likely to be detected. An additional conclusion from these experiments is that the giving of fat along with protein does not impair N balance. This excludes the possibility, inherent in our earlier experiments on man and the rat, that separation of diets into protein-fat and carbohydrate-fat portions may affect protein metabolism adversely because of the fat in the protein-fat portions.

#### SUMMARY

1. Adult male rats were first given a diet with the protein fed apart from the carbohydrate and then the same diet with most of the carbohydrate fed along with the protein. The immediate effect of this change in the time of feeding carbohydrate was a considerable improvement in N balance. After a few days, however, N balance returned to its original level. Subsequent separation of the times of ingestion of carbo-



hydrate and protein caused a transient impairment of N balance. It has been concluded that protein utilization is affected by the presence of carbohydrate in the same meal.

2. No change in N balance was observed in experiments involving similar alterations in the time of feeding fat. The giving of fat a short time before the protein meal was also without effect on N balance.

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# THE ROLE OF PYRIDOXINE IN ECONOMY OF FOOD UTILIZATION <sup>1</sup>

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In 1941 Sure and Dichek demonstrated that riboflavin produces a most pronounced effect on economy of food utilization, and that the increases in body gains are derived mainly from fats and to a lesser but appreciable extent from proteins.

## EXPERIMENTAL

In the present communication the authors are reporting on the role of pyridoxine in economy of food utilization. Twenty-four male and 24 female albino rats were depleted of pyridoxine. The criteria for detecting symptoms of pyridoxine deficiency were dermatitis associated with alopecia and rough, sticky hairs (György and Eckardt, '39; Antopol and Unna, '42). We did not encounter any of the convulsive symptoms described by Chick et al. ('40) and by Daniel and associates ('42). In 26 to 59 days 15 rats, 10 males and 5 females, developed the typical dermatitis-alopecia symptoms and were used for this study, which from the start was conducted as a series of controlled feeding experiments. In each pair of animals the positive control, which received daily 25  $\mu$ g of pyridoxine, was allowed the same amount of food consumed by its litter mate, which received no pyridoxine. The food

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intake was balanced 4 times weekly. The animals at the beginning of the experiments were 26 to 30 days of age and each weighed 45 to 48 gm.

Cerecedo and Fay ('44) demonstrated a relationship between pyridoxine and protein. They found rats on a low protein, pyridoxine-deficient diet to be quite resistant to pyridoxine avitaminosis, and that on diets high in casein the acrodynia appeared at an earlier period and was more severe than on diets low in casein. We chose a diet of the following composition in parts per 100: casein,<sup>2</sup> 30; cellu flour, 2; salts no. 1 (Sure, '41), 4; vegetable shortening,<sup>3</sup> 5; cod liver oil, 3; and sucrose, 56. Each animal received daily 6 times weekly, separate from the ration, the following crystalline components of the vitamin B complex: 25  $\mu$ g thiamine, 25  $\mu$ g riboflavin, 25  $\mu$ g niacin, 150  $\mu$ g calcium pantothenate, 3 mg *p*-aminobenzoic acid, 6 mg choline chloride, and 1 mg inositol. The positive control rats received in addition 25  $\mu$ g of pyridoxine daily.

Each pair of animals was sacrificed at the end of the vitamin depletion period (table 1, column 2) when the pyridoxine deficient rats developed the typical dermatitis-alopecia-sticky and rough hair symptoms and were analyzed for protein ( $N \times 6.25$ ), fat, moisture, and ash according to the technique previously described (Sure and Dichek, '41). In addition, 24 male and 24 female rats of the same initial ages and weights were taken one week after weaning from stock diets and analyzed for protein, fat, moisture, and ash. From the chemical compositions and body weights at the beginning and at the end of the experimental periods, the chemical composition of body gains was computed. The results are summarized in table 1.

It is apparent from this table that while there are great variations among the different groups of animals in the period at which symptoms of pyridoxine deficiency began to be evident, and in the rates of growth of the avitaminotic and control animals, the increased gains in body weight per 100

<sup>2</sup> Smaco brand.

<sup>3</sup> Crisco.

gm of food intake of the litter mates which received 25  $\mu$ g of pyridoxine daily are striking in every case. The gain in body weight per 100 gm of food intake, which expressed the re-

TABLE 1  
*Role of pyridoxine in economy of food utilization*  
(*P* = pathological; *RC* = restricted control)

PAIR NO.	PERIOD OF VITAMIN DEPLETION	CHANGES IN BODY WEIGHT	TOTAL FOOD INTAKE	GAIN IN WEIGHT PER 100 GM FOOD INTAKE	CHEMICAL COMPOSITION OF BODY GAINS		
					FAT	ASH	PROTEIN
	days	gm	gm	gm	gm	gm	gm
1 P ♂	26	+ 12	157.3	7.6	+ 0.98	+ 1.46	+ 2.50
RC ♂		+ 46	165.3	27.9	+ 4.15	+ 2.56	+ 10.68
2 P ♂	34	+ 17	236.2	7.2	- 3.20	+ 1.23	+ 3.81
RC ♂		+ 78	257.7	30.2	+ 6.30	+ 2.00	+ 16.90
3 P ♂	33	+ 26	227.6	11.4	- 2.14	+ 1.90	+ 6.22
RC ♂		+ 67	232.6	28.8	+ 8.10	+ 3.48	+ 15.10
4 P ♂	42	+ 18	272.8	6.6	- 0.06	+ 2.42	+ 6.82
RC ♂		+ 64	288.2	22.2	+ 10.43	+ 3.59	+ 15.08
5 P ♂	40	+ 50	298.3	16.8	- 1.70	+ 2.20	+ 11.58
RC ♂		+ 90	303.0	29.7	+ 11.18	+ 3.40	+ 17.62
6 P ♀	47	+ 23	295.0	7.8	- 0.32	+ 1.17	+ 6.41
RC ♀		+ 54	302.0	17.9	+ 11.15	+ 2.55	+ 11.65
7 P ♂	49	+ 42	338.4	12.4	- 2.44	+ 2.80	+ 11.81
RC ♂		+ 95	346.0	27.4	+ 7.54	+ 4.72	+ 25.60
8 P ♂	47	+ 33	275.6	12.0	- 2.70	+ 1.90	+ 7.89
RC ♂		+ 83	278.7	29.7	+ 3.99	+ 3.29	+ 15.80
9 P ♂	51	+ 23	300.0	7.7	- 3.70	+ 2.34	+ 9.58
RC ♂		+ 82	301.0	27.2	+ 7.31	+ 3.16	+ 16.57
10 P ♂	52	+ 62	381.4	16.3	+ 4.52	+ 3.29	+ 15.12
RC ♂		+ 98	381.2	32.6	+ 10.91	+ 3.42	+ 22.96
11 P ♂	51	+ 65	384.3	16.9	+ 1.24	+ 3.32	+ 12.03
RC ♂		+ 98	391.9	25.0	+ 7.76	+ 4.93	+ 23.07
12 P ♀	52	+ 43	366.3	11.7	- 0.48	+ 2.08	+ 10.47
RC ♀		+ 70	375.2	18.7	+ 12.17	+ 3.30	+ 15.97
13 P ♀	59	+ 60	431.3	13.9	+ 3.64	+ 2.71	+ 14.34
RC ♀		+ 89	427.1	20.8	+ 7.38	+ 3.88	+ 21.65
14 P ♀	54	+ 23	282.5	8.2	- 3.31	+ 1.80	+ 6.71
RC ♀		+ 55	278.4	19.8	+ 6.12	+ 2.52	+ 11.99
15 P ♀	63	+ 65	427.5	15.2	+ 6.09	+ 3.00	+ 14.90
RC ♀		+ 90	428.3	21.0	+ 7.36	+ 4.25	+ 21.43
Average P		37.5	325	11.4	- 0.24	+ 2.24	+ 9.34
RC		75.9	312	25.2	+ 8.12	+ 3.40	+ 17.47

quirements for maintenance and growth, was the yardstick we used for evaluating economy of food utilization.

Averaging the results for 15 paired feeding experiments on the same food intake, 25  $\mu$ g of pyridoxine daily produced a 121% increase in economy of food utilization, an increase of over 800% in fat synthesis, an increase of 86% in protein synthesis, and an increase of 51% in the minerals in the animal body.

#### SUMMARY

Pyridoxine produces a marked effect on economy of food utilization. The increases in body gains are due largely to fat and protein synthesis and, to a less but appreciable extent, to increases of minerals in the animal body.

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# A FATAL VITAMIN E DEFICIENCY DISEASE IN RATS CHARACTERIZED BY MASSIVE LUNG HEMORRHAGE AND LIVER NECROSIS<sup>1</sup>

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SEVEN FIGURES

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Recent reports have ascribed to vitamin E an important role in preventing fatal liver necrosis in rats. Schwarz ('44) found that rats died with extreme liver damage after about 6 weeks on a diet containing 16% of alkali-treated casein. The liver damage and death were prevented by alpha-tocopherol, or by replacing the alkali-treated casein with casein purified by the usual methods. The damage to the liver appeared to be the massive hepatic necrosis first produced by Himsworth and Glynn ('44) as a syndrome distinct from diffuse hepatic fibrosis, and preventable by cystine or methionine.

György and Goldblatt ('49) noted that massive hepatic necrosis developed in about 40% of adult rats kept on a low casein diet when lard was included, but in later experiments less damage was seen when commercial hydrogenated cottonseed oil<sup>2</sup> was the fat source. This difference in results was

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<sup>2</sup>Crisco.

attributed to the tocopherol content of the cottonseed oil. Direct experimental comparison of these results with the influence of tocopherol given rats on the low casein-lard diet was tried in one experiment by these investigators. The result, while suggestive, was difficult to evaluate since necrosis was seen in only three of the 18 rats not receiving tocopherol. In a second experiment with yeast as the source of protein, the tocopherol effect was clearly evident.

The observations of Schwarz ('44) relating to alkali-treated casein could not be duplicated by György and Goldblatt ('49), although an apparently identical experimental technique was used. Hove ('46a, '49) twice tried to repeat the findings of Schwarz, using both albino and hooded strains of rats, but was unsuccessful in both cases. Furthermore, Hove ('46b) reported the absence of gross liver damage in adult rats maintained for 20 weeks on a 5% casein, vitamin E-low diet, although alpha-tocopherol was dramatically effective in conserving body weight.

In view of the negative and variable results obtained previously, it came as a surprise to note in the experiments presently reported the death of a majority of the rats restricted to a 10% casein, vitamin E-low diet, while complete protection was given by vitamin E. Necrosis of the liver was seen in nearly all of these rats. Of great significance may be the observation that death was accompanied, and possibly caused, by massive lung hemorrhage.

## EXPERIMENTAL

### *Diet and animals*

The vitamin E-low diet (E-20) used for most of the work herein described had the following percentage composition: water-washed casein 10 (Salmon, '47), sucrose 56, salt mixture 4 (Salmon, '47), lard 19, cod liver oil U. S. P. 1, vitamin premix 10. The latter consisted of pure vitamins mixed with sucrose to supply the following levels per gram of diet: thiamine, riboflavin and pyridoxine, 5 µg each; calcium panto-



thenate 20  $\mu$ g; niacin 40  $\mu$ g; choline chloride 2 mg; *i*-inositol 0.2 mg; and menadione 2  $\mu$ g.

In one of the experiments oxidized casein was used as the protein source. This was prepared by a method similar to that described by Toennies ('42) but differing in that 3% hydrogen peroxide was used instead of the recommended 30%. The more dilute hydrogen peroxide is equally effective in destroying methionine and tryptophan, and the reaction proceeds more smoothly and with less terminal foaming. The water-washed casein of the basal E-low diet was replaced by oxidized casein at a 16% level and the diet was supplemented with 0.3% of DL-tryptophan. The sucrose level was reduced in order to maintain all other ingredients at standard levels.

Over a period of a year, litters of hooded rats of the Alabama Experiment Station's high-choline-requiring strain were obtained from the stock colony at three weeks of age and at 40 to 50 gm in body weight. These were placed on the basal E-low diet (E-20), with about a third of each litter receiving 7 mg of dl-alpha-tocopherol weekly as an olive oil solution given orally. In more recent experiments dl-alpha-tocopherol acetate was added to the cod liver oil at a level to furnish 1 mg of vitamin E per 10 gm of diet. The experiments were continued until the death of the experimental animals, or for at least 100 days.

## RESULTS

### *Description of the acute vitamin E deficiency*

Over 75% of the male rats died after an average of 10 weeks on the vitamin E-low, 10% casein basal diet. No deaths occurred among the control rats receiving alpha-tocopherol (table 1). Female rats appeared to be much less sensitive to the lack of vitamin E, in that only 5 out of 15 females died on the basal E-low diet. These are not included in the averages given in table 1.

Prior to death the existence of a vitamin E deficiency was evidenced by the blanching of the incisor teeth and by the somewhat slower growth rate. In 5 cases in which the death

process was observed, the rats expired in convulsions. Aside from a few blood flecks about the nostrils, no external evidence of hemorrhage was seen. At necropsy a high incidence of massive lung hemorrhage and severe liver damage was noted. The subcutaneous blood vessels were very prominent. The bladder was distended in nearly every case and contained a large amount of loose sediment. The testes were somewhat smaller than those of the controls receiving tocopherol. The

TABLE 1  
*Fatal vitamin E deficiency among weanling male rats fed the 10% casein basal diet (E-20)*

CATEGORY OF INTEREST	SUPPLEMENTS OF ALPHA TOCOPHEROL	
	None	7 mg weekly
Number of rats	51 <sup>1</sup>	32
Number of spontaneous deaths	37	0
Average number of days on diet	71	100
Liver (% of body weight)	5.0	4.2
Liver fat (crude ether ext., % fresh basis)	3.8	4.4
Testes (% of body weight)	1.7	1.9
Number of rats with gross liver damage	31	0
Number of rats with massive lung hemorrhage	28	0
Growth rate for 6 weeks (gm/rat/day)	0.9	1.3

<sup>1</sup> Six of these rats were removed from experiment between the 8th and the 12th weeks to determine whether the liver and lung lesions were progressive. The remaining non-fatalities are still on the diet after more than 250 days.

stomach, intestines, kidney, and spleen showed no grossly evident lesions. The abdominal cavity was free from excessive fluid.

As is illustrated in figure 1, the lungs were engorged with blood in 28 of the 37 fatal cases (table 1). This condition was extensive enough to be considered the cause of death in these rats. The heart was normal grossly except for some congestion of the coronary vessels, and in two cases scar tissue was evident. The large vessels appeared somewhat distended.

The liver was grossly damaged in 31 of the 37 fatal cases. The surface was smooth but showed a marble-like mosaic

caused by dark and light areas ranging up to 1 cm in width and involving all lobes. In extreme cases the surface of the light-colored areas was depressed. The weight of the liver relative to body weight was somewhat greater than that of the controls receiving vitamin E. The average fat content (fresh basis) of 6 damaged livers was 3.8%, while for the tocopherol-supplemented rats the liver fat was 4.4% (table 1). The moisture content was 69.1 and 69.8%, respectively.

The thymus gland was invariably rust colored, with small dark areas. The brain was normal in the gross. The posterior lobe of the pituitary was rust colored in a few cases.

### *Histological description of the lesions*

The lung tissue of the rats that died due to acute vitamin E deficiency retained its normal architecture, but the inter-alveolar tissue was packed with red blood cells. There was marked distention of the large and small blood vessels. In some areas the alveoli were filled with red blood cells, as is shown in figure 2; for comparison, a section of lung from a control rat is shown in figure 3. There was no evidence of lung infection. The lack of organization of the red blood cell accumulations indicated that this condition was acute and could have been the cause of death in the animal. The peripheral subcutaneous blood vessels were also distended, as is shown in figure 1.

Microscopic studies of the slides prepared from the livers of the vitamin E-low rats revealed an extensive necrosis of centrilobular origin. The nature and extent of the necrosis is shown in figure 5. In most cases this type of necrosis occurred extensively in all lobes of the liver. In less widespread cases the damage was confined to smaller areas of certain lobes, and some lobes were fairly normal in appearance. In 7 cases entire lobes of the liver seemed to have undergone massive necrosis (fig. 6), and other lobes of the same liver showed patchy centrilobular necrosis with large areas of normal tissue. In three of the experimental animals large areas of the liver parenchyma were completely de-

stroyed and in some areas regeneration had occurred, which resulted in changes in the architecture of the liver, as is shown in figure 7. The livers of the control rats receiving vitamin E were normal (fig. 4). There was no indication of significant fat accumulations in these livers. No significant pathologic changes were observed in microscopic examination of tissues taken from other organs.

TABLE 2

*The relation of various supplements to the fatal vitamin E deficiency disease*

VARIATION IN LOW CASEIN DIET	NUMBER OF:		TIME OF DEATH	GROWTH RATE IN 6 WKS.
	Rats	Deaths		
			days	gm/day
Added casein (to 16%)	20	0	..	3.9
DL-methionine, 0.1%	5	4	58	1.3
L-cystine, 0.1%	5	2	85	1.6
Theophylline, 0.1%	5	5	54	0.7
Low calcium <sup>1</sup>	4	4	63	0.1
Low potassium <sup>1</sup>	4	4	58	0
High calcium <sup>2</sup>	4	2	86	0.2
High potassium <sup>2</sup>	4	4	65	0.5
None	4	3	67	0.8

<sup>1</sup> Salts of these elements were omitted from the diet.

<sup>2</sup> The carbonates of these elements were added to the diet at the expense of sucrose to double the normal supply.

*The relation of protein to the fatal vitamin E deficiency*

When the casein in the vitamin E-low diet was increased to 16% at the expense of the sucrose, all other ingredients remaining constant, death and the hemorrhagic syndrome did not occur. A large group of rats have been maintained for 15 months on such a diet, and the only benefit resulting from the vitamin E supplement given to the controls has been the maintenance of normal tooth color, better muscle tone, and greater maximum body weight.

Little if any benefit was derived from the addition to the low casein diet of L-cystine or DL-methionine at levels of 0.1%. The data shown in table 2 indicate that death with

typical symptoms ensued, although the severity of the condition may have been lessened somewhat by the cystine addition. It may also be noted in table 2 that theophylline was completely inactive. Neither the omission nor the doubling of the calcium or potassium content of the diet affected the course of the fatal vitamin E deficiency (table 2).

Data confirming the non-effectiveness of DL-methionine in preventing the fatal vitamin E deficiency are given in table 3. On a diet containing 16% of oxidized casein and supplemented with 0.3% DL-tryptophan, young rats lost weight

TABLE 3

*The occurrence of the fatal vitamin E deficiency on a 16% oxidized casein diet supplemented with methionine and 0.3% DL-tryptophan*

DL-METHIONINE ADDED TO DIET	ALPHA TOCOPHEROL SUPPLEMENT					
	None			7 mg weekly		
	Number of rats	Number of deaths	Growth rate (6 wks.)	Number of male rats	Number of deaths	Growth rate (6 wks.)
%			gm/day			gm/day
0	3	3 <sup>1</sup>	— 0.6	3	3 <sup>1</sup>	— 0.7
0.25	6	3	0	4	0	0
0.50	3	3	0.9	3	0	1.3
1.00	3	2	1.6	3	0	2.0

<sup>1</sup> The rats in these groups were all dead by the 5th week.

rapidly and died in 4 to 5 weeks. Vitamin E supplements did not influence this course. None of these rats showed gross pathological lesions of the liver, lungs, or other organs. With 0.25% of DL-methionine added to the diet, body weight was maintained but no growth occurred. Three of the 6 rats without vitamin E died before the 10th week on this diet; typical lung and liver lesions were present. With 0.5% of methionine added to the diet, growth was considerably better, especially with supplements of alpha-tocopherol; but all rats without vitamin E died with typical vitamin E deficiency lesions between the 8th and 10th weeks. With 1.0% of methionine added to the diet, growth was even better; but death

still occurred among the vitamin E-free group. It is to be noted that this diet contained no cystine.

*The effect of the dietary fat level on the fatal vitamin E deficiency*

The basal diet used in the preceding work contained 20% fat. To determine the effect of the dietary fat level on the incidence and severity of the fatal vitamin E deficiency, an experiment was set up using diets with fat levels ranging from 5% to 40%. Groups of 4 weanling male rats were placed on each of these diets. The results, as given in table

TABLE 4

*The relation of the fat content of the 10% casein diet to the fatal vitamin E deficiency in weanling male rats*

DIETARY FAT <sup>1</sup>	NUMBER OF:		AVERAGE TIME OF DEATH	AVERAGE GROWTH RATE IN 4 WKS.
	Rats	Deaths		
%			days	gm/day
5	4	2	59	1.5
10	4	3	53	1.3
20	4	3	62	1.1
40	4	4	58	0.9

<sup>1</sup> The fat in the diet consisted of 1% cod liver oil with the remainder lard.

4, show that the fat level in the diet did not have a profound influence on the incidence and severity of the fatal vitamin E deficiency. The casein levels of the diets were all kept at 10% and not balanced isocalorically with the fat level; this may account for the higher growth rate at the lower fat levels.

#### DISCUSSION

The development of fatal vitamin E deficiency in rats depends upon the simultaneous deficiency of some substance present in casein. Schwarz ('44) removed the casein co-factor by an alkali extraction, and though this technique has been unsuccessful in this laboratory, similar results have been obtained by using a low (10%) dietary level

of water-washed casein. Cystine and methionine appear not to be the sole factors present in casein. However, it should be emphasized that supplements of cystine or methionine higher than 0.1% in the 10% casein diet were not tried; at this level cystine appeared to give some protection, but methionine was inactive. Using higher levels, György and Goldblatt ('49) noted partial protection by either cystine or methionine, while Himsworth and Glynn ('44) obtained complete protection with cystine added to a basal diet containing traces of alpha-tocopherol furnished by peanut oil. In previous work Hove ('48) noted that methionine added at a 0.1% level to a diet of the same composition as that used in the present work was able to replace alpha-tocopherol for the protection of rats against death due to an acute  $\text{CCl}_4$  toxicity. Theophylline was also a good protector against  $\text{CCl}_4$ , but in the present work, at the same dietary level, it was of no benefit. Schwarz ('44) obtained no protection against the fatal liver damage from either cystine or methionine but he did observe that xanthine could replace alpha-tocopherol in this function.

The precise interrelation of the sulfur amino acids, purine bodies, and alpha-tocopherol as they relate to liver damage, lung hemorrhage, and organic solvent toxicity is confused at present. A considerable amount of further study is required. The question may be raised of whether rats restricted to a 10% casein basal diet have, in fact, developed a true vitamin E deficiency. The multiplicity of symptoms ascribed to a lack of vitamin E in various species makes this difficult to answer; but if blanching of the incisor teeth and a slight retardation in the growth rate are accepted as valid criteria, then a vitamin E deficiency can be said to develop within the first few weeks on the diet. This has been observed previously by Hove and Harris ('47).

The ultimate cause of death in the fatal vitamin E deficiency is unknown. Either the massive lung hemorrhage or the liver necrosis may have been sufficient to produce death. The absence of repair processes and fibrosis in the hepatic architec-

ture except in a few cases, and the normal nature of the lungs of animals killed for examination, indicate that the reaction progresses very rapidly. A disorder of vascular origin that limits the blood supply to the liver causes liver necrosis, as has been noted by Himsworth ('47). The occurrence of a massive lung hemorrhage also emphasizes the possibility of a primary vascular disorder.

Mason ('43) has investigated the cause of death of the fetus in the vitamin E-deficient rat. His description is as follows: "Prior to exitus there appear abnormalities in the vascular system characterized by variable degrees of dilation and thrombosis of peripheral and deeper vascular channels and by either localized or diffuse areas of hemorrhage . . . ." This description fits equally well the pathology noted in rats dying of the fatal E deficiency, and supports the conclusion that the basic function of vitamin E is the same whether in preventing the death of young rats *in utero* or in protecting young growing rats against the fatal deficiency, when they are conditioned by a low casein intake.

#### SUMMARY

1. Young rats died of an acute vitamin E deficiency when restricted to a 10% casein diet. Death occurred suddenly in over 75% of the male rats between the 6th and 12th week, or after an average of 71 days on the diet.

2. Death was characterized by massive lung hemorrhage, centrilobular or massive necrosis of the liver, and distention of the subcutaneous blood vessels.

3. Protection against the fatal disease was given by alphatocopherol or by increasing the casein to a level of 16% in the diet. The disease occurred when the casein in the diet was replaced with 16% oxidized casein plus 0.3% tryptophan and up to 1% methionine. Cystine was slightly protective, but neither methionine nor theophylline was of benefit when included in the 10% casein diet at a 0.1% level.



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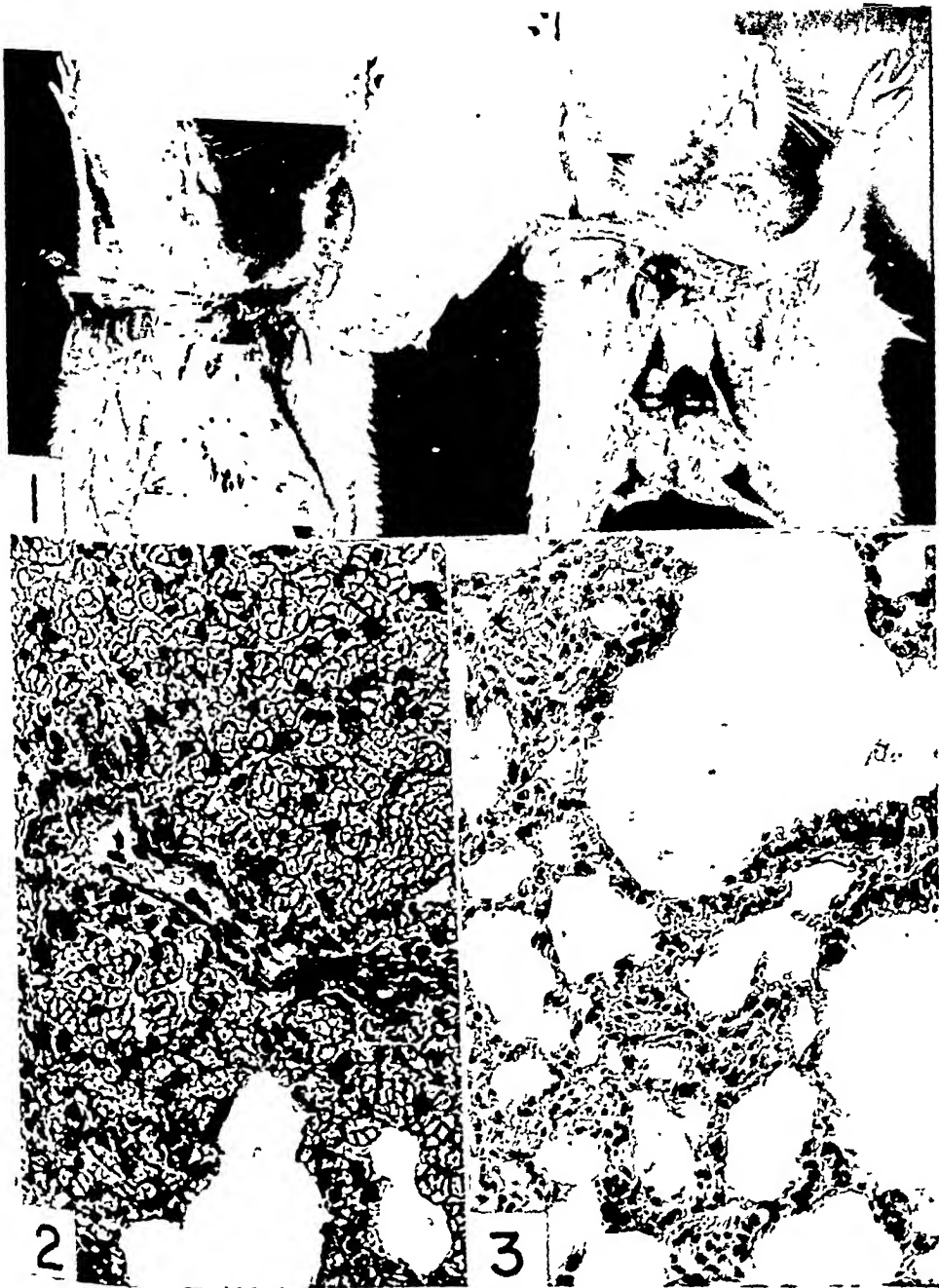
## PLATE 1

### EXPLANATION OF FIGURES

1 Gross dissection showing the massive lung hemorrhage, distended subcutaneous blood vessels, and blanched teeth of a vitamin E-deficient rat (left) which died after 10 weeks on Diet E-20, as compared to the normal appearance of a litter-mate control receiving vitamin E (right).

2 Photomicrograph of the lung of a vitamin E-deficient rat, showing extensive accumulation of red blood cells in the alveolar spaces and interalveolar tissue. Hematoxylin and eosin stain,  $\times 470$ .

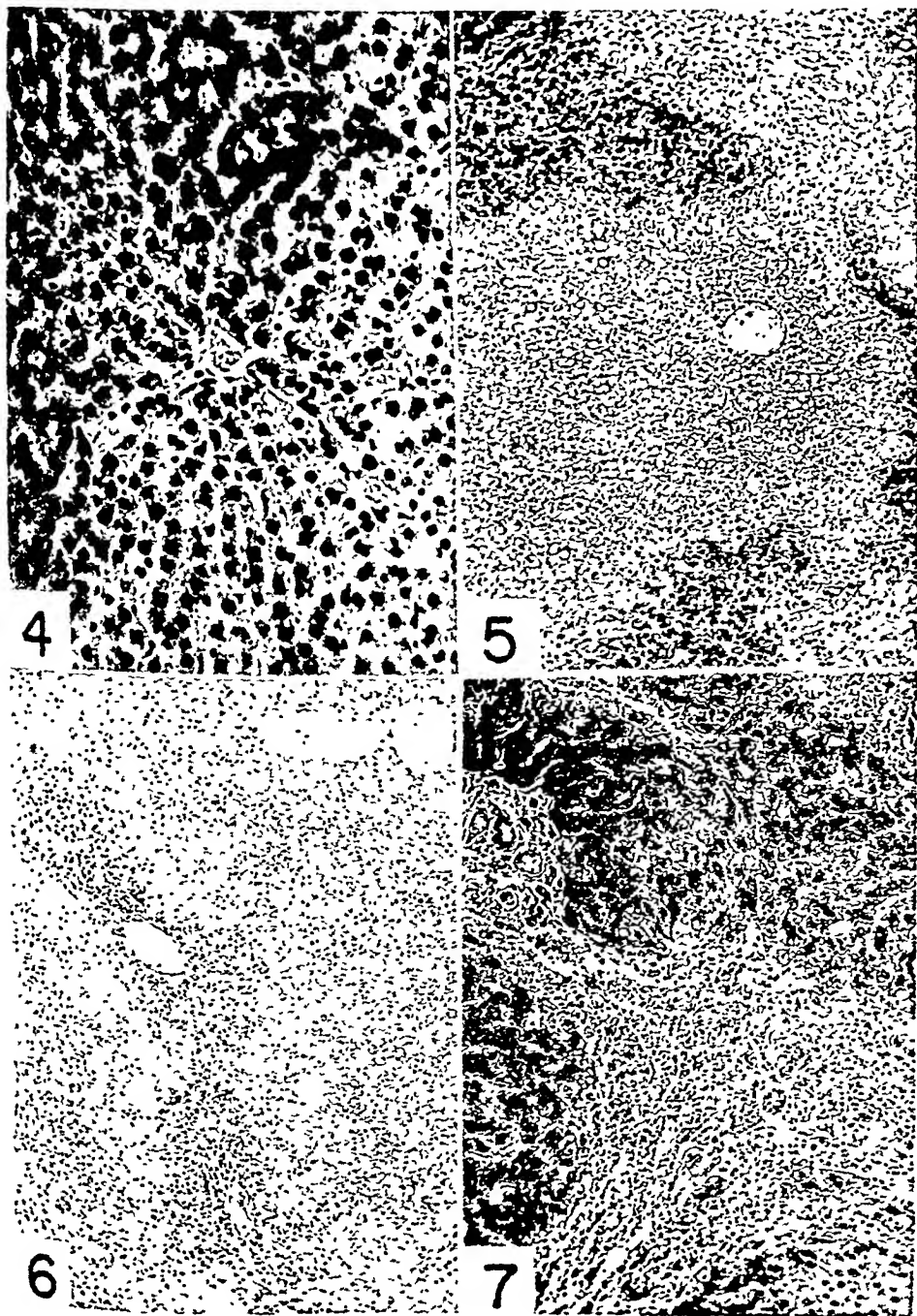
3 Photomicrograph of lung of control rat showing normal histology. Hematoxylin and eosin,  $\times 230$ .



## PLATE 2

### EXPLANATION OF FIGURES

- 4 Photomicrograph of normal liver of control rat receiving vitamin E. Hematoxylin and eosin stain,  $\times 180$ .
- 5 Photomicrograph showing centrilobular necrosis in liver of a vitamin E-deficient rat. This condition appeared in 31 cases. Hematoxylin and eosin,  $\times 90$ .
- 6 Photomicrograph showing massive necrosis in the liver of an E-deficient rat. This condition appeared in 7 cases. Hematoxylin and eosin,  $\times 90$ .
- 7 Photomicrograph of an area of the liver of a vitamin E-deficient rat, showing regeneration of liver and changes in architecture. This condition appeared in three cases. Hematoxylin and eosin,  $\times 110$ .



and in metabolism. The preparation of a food for consumption by animal or man, by commercial processing or culinary practices, has been repeatedly shown to modify the completeness of utilization of protein in digestion or in metabolism, or in both. The effect may be favorable but is generally unfavorable, while the most effective agent in whatever effect is produced is heat, modified by the amount of water present in the food. Obviously the effect of heat will depend upon the temperature attained within the food and the time of exposure. There is still much to be learned concerning the effect of heat processing on the nutritive value of food proteins, which is merely one phase of the much larger problem of the total effect of heat processing on foods, including in particular its effect on the contained vitamins.

This report will be concerned with: (1) the changes in the nutritive value of the proteins of peanut meal, cottonseed flour, linseed meal and corn brought about by their subjection to commercial processes commonly used in their preparation for animal or human use; (2) the changes induced in the proteins of sunflower seed flour by autoclaving in the laboratory for 30 min. at 20 lb. steam pressure; and (3) the changes in the protein value of beef round brought about by roasting according to a standard method.

Total heat damage ranging from 10 to 20% in the net utilization of protein by the growing rat was demonstrated for the proteins of the peanut, corn, sunflower seed, and cottonseed, generally in protein digestibility rather than in biological value. Beef proteins were not appreciably injured by roasting, while the proteins of flaxseed were improved slightly in nutritive value, mainly because of an improvement in digestibility.

#### EXPERIMENTAL PROCEDURES

The heated and unheated, or very mildly heated, food products were compared with respect to the nutritional value of their content of conventional protein ( $N \times 6.25$ ) by nitrogen metabolism studies with young albino rats weighing initially about 50 gm. The experiments were carried out by a method

developed in this laboratory (Mitchell, '24; '44) leading to the computation of coefficients of true digestibility, making due allowance for the metabolic fecal nitrogen, and of biological values, expressing the percentage of the absorbed nitrogen used for the replacement of endogenous losses and for growth. Generally an experimental group consisted of 5 pairs of rats, paired for equality of sex and litter membership and approximate equality in initial body weight. Throughout the three experimental periods, pair mates received the same amount of their respective diets in a reversal system of feeding whereby each rat in any one experiment received samples of both the unheated and heated product in different experimental periods, 5 rats in one order and the other 5 in the reverse order. The heated and unheated products were tested in periods 1 and 3, while in period 2 all rats received the standardizing diet containing 4% of whole egg protein; in this period the ratio of metabolic fecal N to air-dried food, and the ratio of minimum endogenous urinary N to the three-fourths power of the body weight, were determined. An experimental period lasted from 11 to 14 days, with collections of feces and urine during the last 7 days.  $\text{Fe}_2\text{O}_3$  was used as a feces marker.

The experimental rations contained: a complete salt mixture 4%, NaCl 1%, wheat germ oil 0.5%, cod liver oil 1.5%, and the test food to provide 10% of protein, or as large an amount as possible, with lard added to raise the fat content to 12%, wood flock to raise the fiber content to 2%, and glucose, starch and sucrose to make up to 100%.

#### *The effect of heat processing on peanut proteins*

One thousand pounds of No. 2 Spanish peanuts were treated by the Anderson expeller process at an oil extraction mill in Enterprise, Ala. The peanuts were shelled, ground and heated to 246°F. before entering the expeller. The run in the expeller lasted 8 hours and 6 samples for biological assay were taken during the last 4 hours of the run. The temperature of the barrel at the discharge end was 210°F.

In addition, a sample of the same kind of peanuts from the same source was shelled, ground, and extracted with ethylene dichloride at the plant of the VioBin Corporation, at Monticello, Ill., at temperatures ranging from 75° to 80°C.

The samples will be referred to as No. 0, the solvent extracted sample, and Nos. 1 to 6, the successive samples taken from the Anderson expeller. In preliminary paired-feeding tests, samples 0 and 4, 1 and 5, 2 and 6, 0 and 2, 1 and 4, and 3 and 6 were compared by a method of feeding for equal gains and of diluting the better ration with a nitrogen-free mixture; this method has been described by Mitchell, Burroughs and Beadles ('36). At the end of each experiment in which this technique is employed the two rats in each pair have gained the same in body weight through a feeding period of 5 to 7 weeks on the same caloric intake but on different intakes of protein if the proteins under test differ appreciably in nutritive value.

Only in the comparison of samples 0 and 4 and 2 and 6 were the results significantly different, favoring the lower numbered sample. In the first of these cases the average grams gain in body weight per gram of protein consumed were 1.46 and 1.31, respectively, with a probability of 0.021 (Student, '08) that an average difference as great or greater than this (and in the same direction) would have been brought about by a random combination of the uncontrolled factors in the experiment. In the second case the average protein efficiency ratios were 1.33 and 1.16 in favor of sample 2, the probability being 0.0020 that uncontrolled factors acting at random would have produced a difference as great or greater than this favoring the 2 sample.

These paired-feeding results indicate that heat damage to the peanut proteins had occurred to some small extent in the first samples removed from the expeller, but that this damage had not been aggravated by continued exposure to the expeller temperature up to a limit of 8 hours. It may be pointed out that results of weight gain experiments, even with controlled feeding, cannot be considered conclusive evidence of



the differential nutritive effects of proteins; first, because of possible differential activity of the animals on the two comparative rations, and, second, because of possible differences in the protein content of the gains.

Nitrogen metabolism studies were carried out in a comparison of the solvent-extracted peanut meal, No. 0, and peanut meal No. 1. The average results, with their statistical analysis, are given in the first line of table 1. Evidently the heat incident to the processing of sample No. 1 definitely depressed the biological value of the protein; there is also a suggestion that the digestibility of the protein was slightly increased. The total effect of the heat treatment, expressed as the percentage change in the net utilization of the protein (coefficient of digestibility multiplied by the biological value and divided by 100), was a depression in protein utilization of 5.4% ( $P \pm 0.0037$ ).

A comparison by this method of peanut meals 3 and 6 failed to reveal any difference either in the digestibility of the proteins or in their biological values, although the biological values obtained for both samples, averaging 49.8 for meal 3 and 50.5 for meal 6, were distinctly lower than the average biological value for meal 1, 56.8. The average digestibility of the protein in meal 3 was 94.0, and that in meal 6, 93.2. The average net utilization of protein in the pooled data for peanut meals 3 and 6 was 46.9, compared with an average of 57.7 for the solvent-extracted meal. If these averages may be considered directly comparable, they reveal an average difference in heat damage to peanut proteins of 18.7% between meals 0 and 3.

Mitchell, Burroughs and Beadles ('36) studied the change in protein utilization in peanuts during commercial roasting, when the peanuts were heated to between 400 and 450°F. for 30 to 35 min. The average coefficients of true digestibility of protein for raw and roasted peanuts were 97.4 and 96.1, respectively, and the average biological values of the absorbed protein were 59.4 and 55.1, respectively. Applying Student's ('08) method for the analysis of paired differences, the mean dif-

TABLE 1

*The results of the nitrogen metabolism studies*  
*(Each value is the average of 10 determinations, with the exception noted)*

PROTEIN SOURCE	UNHEATED MATERIAL		HEATED MATERIAL		SIGNIFICANCE OF AV. DIFFERENCE IN NET PROTEIN UTILI- ZATION DUE TO HEAT <sup>1</sup>		TOTAL HEAT DAMAGE TO PROTEIN	
	Digesti- bility	Bio- logical value	Digesti- bility	Bio- logical value	Digesti- bility	Bio- logical value	Total	Signifi- cance
Peanut meal <sup>2</sup>	94.8	60.8	95.7	56.8	P <sup>2</sup> =	P <sup>2</sup> =	%	P <sup>2</sup> =
Sunflower seed meal <sup>4</sup>	93.8	57.1	91.3	52.0	0.08	0.00004	5.4 <sup>3</sup>	0.0037
Cottonseed flour <sup>5</sup>	89.7	61.5	84.6	58.1	0.0015	0.0039	10.2	0.0046
Linseed meal <sup>6</sup>	84.8	69.2	87.5	72.2	< 0.001	0.0034	10.7	0.00004
Corn <sup>7</sup>	90.0	59.6	75.8	56.4	0.022	0.060	—6.6	0.031
Beef round <sup>8</sup>	99.6	73.8	99.3	74.8	< 0.00001	0.055	20.1	0.00044
					insignificant		—1.0	0.27

<sup>1</sup> Net protein utilization is the product of the coefficient of true digestibility and the biological value divided by 100.  
<sup>2</sup> The probabilities in this table are computed according to Student (708).  
<sup>3</sup> Solvent-extracted peanut meal versus "expeller," sample 1.  
<sup>4</sup> Solvent-extracted sunflower seed meal versus autoclaved meal.  
<sup>5</sup> Solvent-extracted cottonseed flour versus meal prepared by the hydraulic process.  
<sup>6</sup> Solvent-extracted linseed meal versus expeller-processed meal. Each value in this test is an average for 5 rats.  
<sup>7</sup> Unprocessed corn versus flaked corn.  
<sup>8</sup> Raw versus roasted beef.  
<sup>9</sup> See the text for further data on extent of heat damage to peanut proteins.

ference in digestibility is highly significant ( $P=0.019$ ), as is the difference in biological value ( $P=0.0023$ ).<sup>1</sup> Computing the total heat damage in this 1936 experiment as was done in the experiment reported in the preceding paragraphs, one obtains a value of 8.3%.

The greater heat damage to the proteins of the peanut in the expeller oil extraction process (18.7%) than in the roasting process (8.3%) is evidently due to the much more prolonged application of heat in the former case.

Buss and Goddard ('48), using the method of protein evaluation proposed by Osborne, Mendel and Ferry ('19), have reported the growth-promoting value of the protein of raw peanuts, peanuts boiled in water for 40 min., peanuts roasted at 320°F. for 40 min., and peanuts roasted at 356°F. for 40 min. The average protein efficiency ratios (grams gain in 8 weeks per gram of protein consumed, with diets containing 10% of protein) were, respectively, 1.74, 1.84, 1.65 and 0.21. No statistically significant differences in growth-promoting value among the first three preparations were established by the assay method used. Lysine supplementation of the peanuts roasted at the higher temperature raised the protein efficiency ratio from 0.21 to 0.93.

### *The effect of autoclaving on sunflower seed proteins*

Solvent-extracted sunflower seed meal was compared, with reference to the nutritive value of the proteins, with the same meal autoclaved at 20 lb. steam pressure for 30 min. The average results, with their statistical analysis, are given on the second line of table 1. This heat treatment significantly depressed both the digestibility of the protein and its biological value, with a total heat damage of 10.2% computed from the drop in net utilization.

<sup>1</sup>In analyzing the data on the biological value of the protein of raw peanuts, the aberrant value of 44 was omitted after application of Chauvenet's criterion. In the paired comparisons the value for the same rat for heated peanuts, 62, was also disregarded.

*The effect of heat processing on the proteins  
of cottonseed flour*

Two cottonseed flours were prepared from the same batch of cottonseed. One was defatted by the hydraulic process, where the decorticated seed is crushed and passed through a series of steam cookers in which the temperature increases progressively from 190 to 240°F. This process takes almost two hours, after which the material is put through a hydraulic press. The other flour was prepared by solvent extraction with ethylene dichloride at a low temperature. The average results of the nitrogen balance studies, with a statistical analysis of the differences between the solvent-processed flour ("unheated") and the hydraulic-processed flour ("heated"), are given in the third line of table 1.

The heat employed in hydraulic processing definitely lowered the digestibility of the protein in cottonseed flour as well as its biological value; the total heat damage, based on the decrease in the net utilization of the protein, averaged 10.7%.

Olcott and Fontaine ('41) have also reported a depression in the nutritive value of cottonseed proteins, in proportion to the time of heat treatment, induced by autoclaving at 17 lb. steam pressure (254°F.). Since the results were obtained by the Osborne, Mendel and Ferry ('19) method, they cannot be compared quantitatively with those contained in table 1.

*The effect of heat processing on the proteins  
of linseed meal*

The proteins of linseed meal were studied by a comparison of two products prepared from the same batch of flaxseed. One product was solvent-extracted at a low temperature with ethylene dichloride, while the other was treated for oil removal by the expeller process. The average results are given in the 4th line of table 1.

With linseed meal, heat improved the digestibility of the proteins ( $P = 0.022$ ), and quite probably their biological value

as well ( $P=0.060$ ). The total improvement averaged 6.6%, with  $P$  equaling 0.031. In this test each value in the table is the average for only 5, instead of 10, rats. The analysis was effected by the computation of Fisher's ('44) "t" value, dividing the  $P$  value by two to put it on the same basis as a Student probability, which always relates to a difference between means in the same direction as that observed.

*The effect of heat processing on the proteins of corn*

In order to test the effect on the nutritive value of the proteins of corn of the processes involved in the manufacture of corn flakes, we secured samples of the unprocessed corn, of the flaked corn and of the final toasted product. In the preparation of these corn flakes, the corn was cut, water was added, and the corn was cooked for from 20 to 45 min. at a steam pressure of 45 to 50 lb. The final toasting process raised the rolled flakes to a temperature of 350 to 400°F. for a period of three to 4 min.

These corn flakes are used mainly in the preparation of dog food; they are not degerminated, as are the corn flakes used for human consumption, although the processing seems to be much the same (Jacobs, '44).

The results of comparing the unprocessed corn with the corn flakes prior to the toasting process (line 5 of table 1) reveal a marked and significant drop in the average digestibility of the protein, from 90.0 to 75.8%, and a smaller drop in the average biological value, from 59.6 to 56.4, a decrease which suggests a heat effect but does not demonstrate it. The over-all heat damage, averaging 20.1%, is highly significant statistically ( $P=0.00044$ ).

The effect of the subsequent toasting of the corn flakes was measured in another nitrogen balance study not reported in table 1. During toasting the protein digestibility increased on an average from 73.8 to 79.8%, the reality of which is attested by the low probability of a chance outcome, 0.00083. The biological value did not change significantly, averaging 55.1 for the untoasted product and 54.3 for the toasted,  $P$

being equal to 0.24. The change in net utilization, + 6.1%, is of doubtful significance ( $P = 0.069$ ), though rather strongly suggestive of a favorable effect of the roasting process.

*The effect of roasting on the protein value of beef*

Two cuts of beef round, 3 in. thick, were taken from symmetrical parts of the same carcass. One was prepared directly for protein assay, while the other was roasted in the food laboratories of the Department of Home Economics by a standard method. The meat was roasted for 5 hours in an oven at 300°F. The medium internal temperature was 160°F. with the thermometer inserted 1.8 in. Both raw and roasted cuts were trimmed of visible fat, ground, dried at a temperature approximating 122°F., reground and then extracted with ether.

The average results of the nitrogen balance studies are given in the last line of table 1. The average digestibilities of protein for the raw and roasted beef and the average biological values are very nearly the same, and statistical analysis of paired differences revealed no grounds for presuming that the method of roasting employed modified appreciably the nutritive value of the protein (nitrogen) for growing albino rats.

Previous reports on the effect of various methods of cooking meat on the nutritive value of the contained nitrogen are cited by Poling, Schultz and Robinson ('44), who found some indications from their own work that roasting fresh pork shoulder may lower the protein efficiency ratio for growing rats when the meat is incorporated in experimental rations at a level of 9 to 10%. In the absence of a statistical analysis of the data it is difficult to judge the validity of this conclusion. As a supplement to a basal diet composed largely of yellow corn, fresh, cooked and cooked-cured meat (Wilder and Kraybill, '47) proved equally efficient in supplementing the lysine deficiency of the basal ration under the conditions of feeding employed. The cooking procedure consisted of heating the

meat in a retort for one hour at a temperature of 234 to 236°F.

*The thiamine and pantothenic acid contents  
of some of the meals studied*

In the hope of detecting a correlation between heat damage to the proteins of food products and the loss of thiamine and pantothenic acid brought about by heating, these vitamins were determined in many of the food products studied with

TABLE 2

*The thiamine and pantothenic acid contents of the  
raw and heated meals*

SAMPLE	THIAMINE	PANTO- ACID THENIC
	$\mu\text{g/gm}$	$\mu\text{g/gm}$
Peanut meal, solvent-extracted	12.0	34.5
Peanut expeller meal 1	7.3	32.5
Peanut expeller meal 2	7.0	37.0
Peanut expeller meal 3	5.1	29.5
Peanut expeller meal 4	5.5	30.5
Peanut expeller meal 5	8.7	38.5
Peanut expeller meal 5	7.4	36.5
Peanut expeller meal 6	6.2	8.5
Flaxseed (linseed)	7.6	11.5
Linseed meal, solvent-extracted	2.4	13.0
Linseed meal, expeller	39.1	15.5
Cottonseed flour, solvent-extracted	16.3	12.0
Cottonseed flour, hydraulic	36.4	12.0
Sunflower seed meal, solvent-extracted		

reference to protein utilization by growing rats. The results are summarized in table 2. The assays were carried out under the supervision of Dr. B. Connor Johnson and by methods that he recommends in his manual (Johnson, '48). The thiamine was determined by a thiochrome method and the pantothenic acid by a microbiological method using *L. arabinosus* as the test organism. It should be noted that the latter method does not determine quantitatively the pantothenic acid bound in coenzyme A.

The thiamine values, more than pantothenic acid values, showed a response to heat, though the correlation with pro-

tein changes was poor, particularly for the peanut "expeller" meals. The destruction of thiamine in cottonseed flour by oil extraction by the hydraulic process was far greater than the decrease in protein value.

#### SUMMARY

The effect of heat as applied to food products during commercial processing and home cooking on the nutritive value of the food proteins for the growing rat has been studied on a number of foods by the nitrogen balance method developed in this laboratory. The samples compared were unheated, or very mildly heated as in a solvent-extraction method, and heated to high temperatures by autoclaving in the laboratory (sunflower seed meal), subjection to oil extraction by the expeller process (peanut and linseed meals) or the hydraulic process (cottonseed flour), or subjection to a process of flaking and toasting (corn). As an example of home cooking, the effect of a standard method of roasting on the proteins of beef was ascertained. In all cases, the unheated or mildly heated sample and the highly heated sample were obtained from the same raw material.

During these heating processes the digestibility of the proteins of sunflower seed meal, cottonseed flour and corn was definitely decreased by amounts ranging from 2.5 to 14.2 percentage units. The biological value of the proteins of peanut meal, sunflower seed meal, and cottonseed flour was also definitely decreased in these processes.

The percentage of total heat damage was highest for corn (20) and peanut meal (18), intermediate for sunflower seed flour (10) and cottonseed flour (11); no heat damage was demonstrated in the roasting of beef. For flaxseed (linseed), heat exerted a favorable effect on protein utilization (7%), especially evident in improved protein digestibility.

The heat processes studied always decreased the thiamine content of those foods subjected to assay, but the pantothenic acid content was not impaired in the peanut meals judged by



an assay method that does not measure the amount of the vitamin contained in coenzyme A.

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# ASSAY OF DRY PROTEINS BY RAT REPLETION METHOD. NUTRITIVE VALUE AND AMINO ACID COMPOSITION OF SIX REF- ERENCE PROTEINS

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ONE FIGURE

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The rat repletion method of assaying protein value has been well established by Cannon and his co-workers at the University of Chicago (Wissler, Steffee, Woolridge, Benditt and Cannon, '47). The qualitative and quantitative requirements of amino acids for tissue repletion (Frazier et al., '47; Cannon, '48) and for maintenance (Wissler et al., '48; Frazier et al., '49) have been reported. The gain in weight of protein-depleted rats fed amino acid mixtures was reported to be directly parallel to changes in carcass protein (Benditt et al., '48; Steffee et al., '49).

The rat repletion method was studied in this laboratory as a means of assaying the essential amino acid value of liquid protein hydrolysates (Frost and Sandy, '48a, '48b). It was subsequently found to be convenient for determining the nutritive value of amino acid mixtures in solution (Frost and Sandy, '49).

The present study deals particularly with the assay of whole powdered protein preparations. As in the assay of protein hydrolysate solutions, the nitrogenous supplement was fed at controlled critical levels of nitrogen apart from

the remainder of the diet. Separate feeding of the supplement is believed to offer certain advantages over incorporation in the diet, as will be brought out in the discussion section of the present paper. Six reference protein materials distributed to various laboratories by the Bureau of Biological Research of Rutgers University provided substances of especial interest for the study. Hawley, Murlin, Nasset and Szymanski ('48) described these 6 proteins in connection with studies on their biological value in humans.

TABLE 1  
*Non-protein diet (NP no. 10) for rat-repletion studies*

COMPONENT	AMOUNT	COMPONENT	AMOUNT
	gm/100 gm		mg/100 gm
Sucrose	83	Thiamine HCl	0.6
Salts—U.S.P. (No. 1)	4	Riboflavin	1.2
CaHPO <sub>4</sub> · 2H <sub>2</sub> O	1	Nicotinamide	3.7
Agar	1.4	Pyridoxine HCl	0.6
Primex	4.2	d (+) Ca pantothenate	5.0
Corn oil	4.6	p-aminobenzoic acid	0.6
Cod liver oil	1.4	Folic acid	0.6
Choline chloride	0.15	Biotin	0.04
Inositol	0.1	Menadione	0.4
Liver fraction "O" (Wilson)	0.1	CuSO <sub>4</sub> · 5H <sub>2</sub> O	4.0
Ascorbic acid	0.01	ZnSO <sub>4</sub> · 7H <sub>2</sub> O	4.0
MnSO <sub>4</sub> · 2H <sub>2</sub> O	0.01	CoCl <sub>2</sub> · 6H <sub>2</sub> O	0.2

#### EXPERIMENTAL

Weanling male rats were raised to a weight of 180 to 220 gm on a stock colony diet preparatory to depletion on a non-protein diet. Diets and water were fed ad libitum throughout the assays. The composition of the non-protein diet (diet NP 10) is shown in table 1. This diet differs from that previously used (Frost and Sandy, '48a, '48b) because of an increased amount of phosphorus and calcium and through the addition of copper, cobalt, manganese, folic acid, biotin, p-aminobenzoic acid, inositol, menadione, and a low nitrogen liver fraction as a source of vitamin B<sub>12</sub>. The diet supplies about 4.2 cal. per gram and contains 0.03% N. Rats have been carried

through 5 successive periods of depletion and repletion on this diet without apparent injury. It is our practice, however, in using rats for repeated assays, to return them to a stock diet at intervals for equilibration.

Near-adult male rats lose an average of 20 to 25% of body weight during the initial 12-day depletion period on a non-protein diet. The rats are ordinarily prepared for assay of either liquid amino acid solutions or dry proteins by a trial period wherein a standard 5% fibrin hydrolysate<sup>1</sup> is fed for three days. Animals which do not learn to drink the full allotment of about 40 ml during this period may be eliminated. Skim milk has been successfully tried in place of the fibrin hydrolysate in the drinking trial period; no water is offered during this time. Animals prepared in this way show great avidity for either dry protein or liquid hydrolysates and seldom fail to consume the allotted supplements. In the assays herein reported, all supplements were consumed completely.

The Rutgers reference proteins were assayed at two levels of nitrogen, i.e., 0.12 and 0.24 gm N per rat per day. These levels of nitrogen, using the protein conversion factor 6.25, correspond to levels of protein in the diet of about 7 and 14%. The upper level is approximately the minimum level of feeding at which proteins of the best biological value elicit a maximum response. The lower level is a highly critical level of nitrogen intake, though somewhat above the absolute amino acid minima required for net repletion of tissue proteins to proceed. Nitrogen balance studies in selected cases served to demonstrate the sharp change from nitrogen loss to strong positive N balance during the shift from depletion to repletion. Benditt et al. ('48) have clearly demonstrated the relation of tissue protein synthesis to body weight gain at various levels of N intake in adult protein-depleted rats, so that further elaboration of our data along this line was not undertaken.

<sup>1</sup> Aminosol, 5%, in dextrose, 5%, was used on the basis of convenience. Other commercial hydrolysates may be suitable.

Groups of 5 rats were used for assay. In a few instances the same groups of animals were used in repeat assays of the same protein in order to establish the duplicability of results. Purified beef blood fibrin and lactalbumin were used as comparison standards. Results are shown in table 2.

TABLE 2  
*Rat repletion responses to selected protein materials at two controlled levels of feeding*

PROTEIN SUPPLEMENT <sup>1</sup>	LEVEL OF FEEDING SUPPLEMENT			
	0.12 gm N/day		0.24 gm N/day	
	Ave. 12-day gain and std. error	Range	Ave. 12-day gain and std. error	Range
	gm	gm	gm	gm
Egg albumin	38 ± 3.5	30-46	80 ± 4.9	72-98
Whole egg (defatted)	33 ± 1.4	30-36	66 ± 3.9	53-81
Beef muscle (defatted)	31 ± 1.4	26-33	46 ± 3.5	39-53
Casein	31 ± 2.9	23-37	45 ± 3.7	35-52
Peanut flour	8 ± 6.3	1-13	32 ± 2.7	25-41
Wheat gluten	9 ± 2.3	5-14	19 ± 2.4	11-26
Fibrin, beef blood	35 ± 2.3	34-45	59 ± 0.7	58-60
Lactalbumin	44 ± 2.1	36-52	60 ± 4.5	45-70

<sup>1</sup> The first 6 protein samples are the reference samples distributed for collaborative trial by the Bureau of Biological Research of Rutgers University and described by Hawley et al. ('48). The fibrin is washed and dried beef blood fibrin, which contains 15% N. The lactalbumin is Borden lactalbumin 1542, which contains 12.5% N.

### *Amino acid analyses*

The Rutgers samples were subjected to microbiological and chemical analysis for essential amino acids so that a direct correlation could be made with the rat repletion response. One-gram samples of the proteins were refluxed with 8N HCl for 8 hours to provide complete hydrolysates for microbiological analysis by the methods of Stokes et al. ('45) and Gunness and associates ('46) for leucine, isoleucine, phenylalanine, threonine, histidine, lysine, arginine, valine, methionine and tyrosine. Tryptophan was determined chemically by the method of Graham et al. ('47). The intact proteins were analyzed for nitrogen by the macro-Kjeldahl procedure

with a mercury sulfate catalyst, for ash, and for moisture. Results of all analyses are shown in table 3. For convenience in comparison, the amino acid values were calculated on the basis of 16% N for all proteins.

As will be seen in the table, the total essential amino acid content of peanut flour and gluten is much lower than for other proteins. There are clear-cut limiting deficiencies of methionine and lysine, respectively, in these two proteins. Limiting amino acids in the other proteins are less apparent.

TABLE 3  
*Amino acid analyses of Rutgers reference proteins*  
(Calculated to basis of 16% N)

	EGG ALBUMIN	CASEIN	WHOLE EGG	PEANUT FLOUR	WHEAT GLUTEN	BEEF MUSCLE
	%	%	%	%	%	%
Arginine	5.1	3.9	6.3	8.6	3.1	7.5
Histidine	2.7	3.4	2.2	2.1	2.0	4.4
Isoleucine	5.8	5.8	5.9	3.4	4.2	4.8
Leucine	9.5	9.8	9.1	5.7	6.5	9.2
Lysine	4.5	7.0	5.7	2.5	1.6	10.0
Methionine	4.1	3.4	3.5	0.6	1.3	3.0
Cystine	3.3	0.4	2.2	1.7	2.7	1.2
Phenylalanine	6.8	5.8	6.0	4.3	4.9	4.9
Tyrosine	4.0	5.5	3.4	2.8	2.8	2.5
Threonine	4.9	4.6	5.2	2.3	2.6	5.6
Tryptophan	2.0	1.4	1.9	1.5	1.3	1.9
Valine	7.9	7.4	7.9	3.7	3.7	6.0
Total	60.6	58.4	59.3	39.2	36.4	61.0
Total Nitrogen	11.8	13.22	12.16	10.16	12.65	15.65
Ash	6.2	0.9	4.4	3.9	0.6	4.2
Moisture	8.9	8.1	5.1	3.9	7.0	3.2

### *Technique for 5-day routine assay*

A convenient assay schedule which places all weighings and supplements in the 5-day work week, and which provides for repeated use of rats, is now in use in these laboratories for routine control of an intravenous fibrin hydrolysate.<sup>2</sup> Ani-

<sup>2</sup> See footnote 1, page 429.

imals prepared as described above are fed test materials for 5 days at a level of 0.2 gm N per day. The rats are fed a stock diet for two days over the week-end for maximum repletion. They are then returned to the non-protein diet for 7-day depletion, after which the assay cycle is repeated. Groups of 5 male rats are used and the assays are controlled by a reference protein, lactalbumin. Water is fed ad libitum. The procedure is applicable to routine control of dry protein materials or of liquid protein hydrolysates.

A curve representing the average weight changes of 5 rats from a control group used through 8 successive assays is shown in figure 1. The average response of the 5 rats, together with the range, is shown. A step-wise regular increase in weight occurs under these conditions despite the periodic depletions. As will be observed, the magnitude of the response gradually becomes somewhat less. The assays are judged in reference to the parallel response of the positive control group, so that this variable is controlled. The assay groups which ran parallel to the group shown in figure 1 occasionally gained an average of only about 12 gm during the 5-day assay period. Lesser gains such as this did not appear to affect subsequent responses. The two-day period on a stock diet is particularly useful in permitting repeated use of the animals because it provides a strong equalizing influence.

#### DISCUSSION

The relative nutritive value of individual proteins for growth, repletion or maintenance is primarily related to their essential amino acid composition. There is also a question of the availability of certain amino acids in raw and processed proteins. None of the proteins presently studied was heated above about 80°C. (Hawley et al., '48). The wheat gluten was made from agenized flour, which has no apparent toxic effect on rats. In general, the proteins studied are considered unchanged from their native state as regards nutritive value.



Fair agreement in the relative rating of the proteins obtains between our results and those of Hawley et al. ('48) and Murlin et al. ('48). It may be pointed out that the studies of the Rochester group were involved with maintenance, requirements for which are quantitatively different than those for repletion. The relatively high requirements of the rat for the sulfur amino acids may account in part for the standout

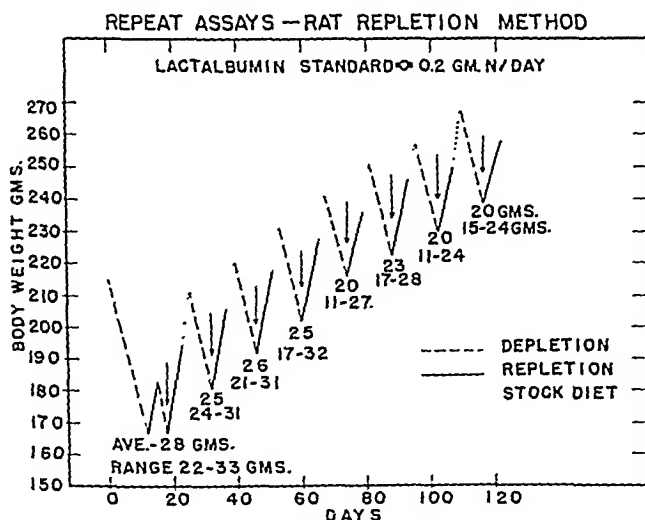


Fig. 1 Average weight changes of 5 rats on lactalbumin as a standard in manufacturing control assay. First part of curve shows 12-day depletion on non-protein diet followed by three-day "drink trial" and three-day redepletion. Subsequent assay cycles consist of lactalbumin (5 days' repletion), stock diet (2 days) and redepletion (7 days). Averages and ranges of weight gain for each of the 8 assay periods are shown.

performance of egg albumin and lactalbumin in the present study over the other proteins high in biological value, particularly fibrin, beef muscle and casein.

Minimum daily requirement values for a maximum rate of repletion in the adult protein-depleted rat have been advanced on a tentative basis by Cannon ('48), as follows: tryptophan 13, phenylalanine 37, leucine 77, isoleucine 66, methionine 35, threonine 45, lysine 51, histidine 22 and valine 53 mg per rat per day. These values were established with a 16-amino acid

mixture by individually reducing the level of each of the essential amino acids, step-wise, to a point where a falling off in the magnitude of repletion response began to take place. According to Cannon, the values are tentative and are, therefore, subject to revision.<sup>3</sup> Also, the values apply precisely only to the 16-amino acid mixture patterned after casein which was used by the Chicago group.

One can calculate the adequacy of each of the essential amino acids as supplied by the various proteins at the 0.12 and 0.24 gm N levels of feeding in comparison with the above minima. In spite of the obvious quantitative limitations, there is good correlation between the nutritive value as herein determined and the calculated adequacy of essential amino acids supplied by the respective proteins. When the necessary calculations are made, it is seen that egg albumin and whole egg contain good patterns of the essential amino acids which approach minimum requirements even at the 0.12 gm N level of feeding. The limiting deficiency of both whole egg and egg albumin at this level appears in the isoleucine content, which supplies in each case only 70% of the requirement for maximum repletion. Obviously, at the higher level of feeding no deficiency is presented by these proteins and maximum rates of repletion are possible. Again, for defatted beef muscle at the lower feeding level 44% of the methionine need and 47% of the isoleucine need are supplied, which would appear to account for the lesser response to this protein. At the lower feeding level the limiting deficiencies for the remaining proteins appear as follows: for casein, methionine 64% and tryptophan 69%; for peanut flour, methionine 13% and lysine 32%; and for wheat gluten, lysine 20% and methionine 26%.

The primary deficits of methionine in peanut flour and of lysine in gluten are well-known. It is of interest to note that the second limiting deficiency for peanut flour appears to be lysine and for wheat gluten, methionine. Little or no supple-

<sup>3</sup> It was learned by personal communication with Dr. Paul R. Cannon that repletion minima revised slightly from those shown above are now in press, together with minimum requirements for maintenance of nitrogen balance.

mentary effect would be predicted, therefore, for a mixture of these proteins. In assessing methionine as a limiting amino acid, one must consider the attendant cystine content, and also the factor of utilization of methionine. The low cystine content of casein is well known and there is evidence in this laboratory (Frost and Sandy, '48b) that the methionine of casein is not completely utilized.

Final assessment of the over-all nutritive value of proteins must also be concerned with metabolic interrelationships having to do with the balance among amino acids themselves and with other nutrients. Such well recognized interrelationships as that between tryptophan and niacin may require increasing attention, with regard to the adequacy of the diet in factors which outwardly appear to be quite unrelated.

Mitchell and Block ('46) and Block and Mitchell ('46) have compared the amino acid composition of a large number of food proteins with that of whole egg as a standard of nutritive excellence. The chemical scores thus obtained are in fair general agreement with the results of growth and nitrogen balance assays in rats. Such methods of chemical scoring are of considerable value in revealing the nature of amino acid deficiencies in proteins. The meaningfulness of the particular reference standard chosen, the correctness of the amino acid analyses, lack of knowledge of species requirements, and the effect of processing on protein value are, however, all questions with which the analyst and nutritionist must cope in any such appraisal of relative protein values. Because these questions involve many variables which are not amenable to control, it is expected that animal assay methods will continue in vogue for some time and that methods of chemical scoring will provide valuable supplementary information.

The repletion method may be considered of particular value in measuring the adequacy of proteins to correct conditions of protein deficit such as occur during growth, pregnancy and lactation, and convalescence from illness or injury. The demands for essential amino acid adequacy and balance are

greatest during these periods, and are fairly well simulated by the conditions of the repletion method. The rat growth method is probably equally valuable, but requires longer time and does not allow repeated use of animals. A criticism of both methods as applied to protein value for human nutrition is pointed up by the difference in species requirements for methionine (Cox et al., '47). Where a limitation in total sulfur amino acids for the rat is known to exist, as in casein, correction can be made by incorporating an appropriate amount of methionine in the basal diet.

Although it is common practice in assaying food substances such as vitamins to feed them separately, proteins have generally been incorporated directly in the diet. Separate feeding of the supplement appears to us to offer advantages in respect to measurement of intake, and also to offer certain theoretical advantages pertaining to the experimental results and the interpretation thereof. In the present method the supplement is fed separately from the remainder of the diet, which is supplied *ad libitum*. Under these conditions, the amount of non-protein diet consumed is thought to be a reflection of appetite, which in turn reflects the well-being of the animal. The results of assays on the same proteins and different proteins at various critical levels of nitrogen are in support of this hypothesis. The quality of a protein may be excellent, but if it is not fed at an optimum level the supply will itself automatically limit the appetite and hence the food consumption. Proteins of poor quality, even when fed at relatively high levels, may still not meet the full quantitative requirements for a maximum rate of repletion, which results in a limited intake of total food. Hegsted and Haffenreffer ('49) have recently presented evidence and a rationale in support of *ad libitum* feeding, particularly in regard to calorie intake. Feeding methods which limit the amino acid nitrogen intake without limiting the intake of other food substances are based on this line of reasoning.

The ranges as well as the standard errors of the repletion responses to the reference proteins are shown in table 2 as

an aid in considering the mathematical significance of the data. In general the standard errors are small considering that only 5 animals were used in each group. The variation in response was greatest for peanut flour and wheat gluten fed at the lower nitrogen level. The latter conditions are most rigorous and it would be expected that even small differences in protein reserves might affect the results between animals. Although such information is of interest, the object of the investigation was not to develop the most rigorous conditions to reveal protein deficits, but rather to develop a simplified method which would reliably classify a wide range of proteins as to essential amino acid value, with a minimum of variance within assay groups.

The "t" test of significant difference was applied to the data of table 2 and a P value of 0.05 or less was considered statistically significant. By this criterion it was found that the difference in response between egg albumin and whole egg is significant at the 0.24 gm N level but not at the 0.12 gm N level. The differences between whole egg and beef muscle (or casein) and between peanut flour and gluten were highly significant at the 0.24 gm N level but not at all significant at the 0.12 gm N level. It would appear from this that the 0.24 gm N level allows better resolution of differences in nutritive value than does the 0.12 gm N level. The level of 0.2 gm N per day was chosen as advantageous for routine control purposes since it provides a good determination of nutritive value at a level of feeding where the ration is readily consumed.

#### SUMMARY

Six reference proteins obtained from Rutgers University as part of a collaborative test program, plus fibrin and lactalbumin, were assayed by the rat repletion method as supplements separate from a non-protein diet. Feeding levels of 0.12 and 0.24 gm N per rat per day were studied. The repletion response to the reference proteins was roughly in the

following order: egg albumin, defatted whole egg, defatted beef muscle and casein about on a par, peanut flour, and wheat gluten. Fibrin and lactalbumin compared favorably with the egg proteins. The weight responses were related to the essential amino acid composition of the reference proteins. The advantages and limitations of the method are discussed, together with the significance of the data obtained.

A convenient method for routine control of the protein value, wherein rats are reused many times, is described.

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## OSBORNE AND MENDEL AWARD

Nominations are invited for the Osborne and Mendel Award of \$1000.00 established by the Nutrition Foundation, Inc. for the recognition of outstanding accomplishments in the general field of exploratory research in the science of nutrition. It shall be given to the investigator who, in the opinion of a Jury of Award, has made the most significant published contribution in the year preceding the annual meeting of the Institute, or who has published a series of contemporary papers of outstanding significance.

The Award will be presented at the annual meeting of the American Institute of Nutrition.

The recipient will be chosen by a Jury of Award of the American Institute of Nutrition. As a general policy, the Award will be made to one person. If, in the judgment of the Jury of Award, an injustice would otherwise be done, it may be divided among two or more persons. Normally preference will be given to research workers in the United States and Canada, but investigators in other countries, especially those sojourning in the United States or Canada for a period of time, are not excluded from consideration. Membership in the Institute of Nutrition is not a requirement for eligibility and there is no limitation as to age.

Nominations may be made by anyone. Nominations for the 1950 Award, accompanied by data relative to the accomplishments of the nominee, must be sent to the Chairman of the Nominating Committee before January 15, 1950.

H. E. CARTER  
*University of Illinois*  
*Urbana, Illinois*

CHAIRMAN, NOMINATING COMMITTEE

## MEAD JOHNSON AND COMPANY 'B-COMPLEX' AWARD

Nominations are solicited for the 1950 Award of \$1000.00 established by Mead Johnson and Company to promote researches dealing with the B-complex vitamins. The recipient of this Award will be chosen by a Committee of Judges of the American Institute of Nutrition and the formal presentation will be made at the annual meeting of the Institute in the spring of 1950.

The Award will be given to the laboratory or clinical research worker in the United States or Canada who, in the opinion of the judges, has published during the previous calendar year, January 1 to December 31, the most meritorious scientific report dealing with the field of the 'B-complex' vitamins. While the award will be given primarily for publication of specific papers, the judges are given considerable latitude in the exercise of their function. If in their judgment circumstances and justice so dictate, it may be recommended that the award be made to a worker for valuable contributions over an extended period but not necessarily representative of a given year. Membership in the American Institute of Nutrition is not a requisite of eligibility for the award.

To be considered by the Committee of Judges, nominations for this award for work published in 1949 must be in the hands of the Chairman of the Nominating Committee by January 15, 1950. The nominations should be accompanied by such data relative to the nominee and his research as will facilitate the task of the Committee of Judges in its consideration of the nomination.

W. H. SEBRELL, JR.  
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CHAIRMAN, NOMINATING COMMITTEE

## BORDEN AWARD IN NUTRITION

Nominations are solicited for the 1950 Award of \$1000.00 and a gold medal made available by the Borden Company Foundation, Inc. The American Institute of Nutrition will make this award in recognition of distinctive research by investigators in the United States and Canada which has emphasized the nutritive significance of the components of milk or of dairy products. The award will be made primarily for the publication of specific papers, but the judges may recommend that it be given for important contributions over an extended period of time. The award may be divided between two or more investigators. Employees of the Borden Company are not eligible for this honor.

The formal presentation will be made at the annual meeting of the Institute in the spring of 1950. To be considered for the award, nominations must be in the hands of the Chairman of the Nominating Committee by January 15, 1950. The nominations should be accompanied by such data relative to the nominee and his research as will facilitate consideration for the award.

L. A. MAYNARD

*Cornell University, Ithaca, New York*

CHAIRMAN, NOMINATING COMMITTEE



# THE EFFECT OF COCOA UPON CALCIUM UTILIZATION AND REQUIREMENTS, NITROGEN RETENTION AND FECAL COMPOSITION OF WOMEN<sup>1</sup>

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Wide interest has been evidenced in the effect of cocoa on the utilization of calcium, particularly that of milk, since Mueller and Cooney ('43) indicated that cocoa consumed with milk may depress the utilization of calcium in the growing rat by 20 to 25% when the proportion of milk solids to cocoa is only 3.3 to 1. Mitchell and Hamilton ('46) confirmed these results when the same brand of cocoa, "a low-cost American process cocoa containing 10.58% fat," was used. The present study was instigated to test the possible effect of a moderate-cost American process cocoa upon the calcium metabolism of humans, the group chosen for study being college women.

## EXPERIMENTAL PROCEDURE

### *General plan*

The study extended from October 10, 1944 to May 31, 1945 and was divided into 11 units during which several levels of

<sup>1</sup> This investigation was aided by funds contributed to the University of Illinois by the National Dairy Council on behalf of the Chocolate Ingredient Manufacturers, CIM, and the International Association of Ice Cream Manufacturers.

<sup>2</sup> Now at the Department of Home Economics, University of California, Los Angeles.

cocoa were fed. Continuous calcium balances (broken only by 15 days for the Christmas holidays) were determined and the experiment was so planned that calcium utilization and requirement values could be calculated. During some of the later periods, nitrogen balances and fecal dry matter were determined.

### *Subjects*

Seven college women, 18 to 22 years of age, served as experimental subjects during the initial part of the study. One withdrew at the Christmas vacation and was immediately replaced by an 8th subject, aged 20, who continued until the end of the study. These subjects were adjudged healthy as the result of an initial physical examination by a local physician. They ranged in weight from 47 to 72 kg and in standing height from 157 to 173 cm.

### *Dietary regime*

(a) *Composition of diets.* A low-calcium basal diet containing an average of 225 mg of calcium and 70 to 80 gm of protein was fed. This diet consisted of 4 basic menus which were repeated in a definite order at 4-day intervals. The following foods were included: beef, ham, bacon, dried beef, gelatin, bread (70% extraction flour, milk-free), macaroni, rice, other cereals, potatoes, peas, corn, cabbage, lettuce, green beans, carrots, applesauce, peaches, apricots, pears, orange juice, tomato juice, grapefruit juice, butter, coffee, cream and sugar. The diet was supplemented daily by 5,000 U.S.P. units of vitamin A and 500 U.S.P. units of vitamin D in capsule form.<sup>3</sup> Caloric intakes were adjusted to individual needs with bread and sugar. Ad libitum consumption of distilled water was allowed. Whole homogenized milk<sup>4</sup> and cocoa powder were added in quantities enumerated in the following section. As these quantities were changed, adjustments were made in other

<sup>3</sup> Natola, Parke-Davis.

<sup>4</sup> A blend of three parts of whole and one part of skimmed milk was used in periods VIII and IX in order to adjust the fat content of the diet.

foods in an attempt to maintain a constant ratio of total calories, protein and fat. The cocoa used in this study was an American process cocoa of medium cost. The cocoa for the entire study was obtained in one lot at the beginning and by analysis had the following percentage composition: dry matter, 97.16; ether extract, 20.22; crude protein ( $N \times 6.25$ ), 22.56; tannic substances, 11.27; ash, 5.26; crude fiber, 3.50; theobromine, 1.79; phosphorus, 0.659; calcium, 0.145; oxalic acid, 0.504. It had an energy value of 5.48 cal. per gram.

(b) *Feeding plan.* The basal diet described above was used throughout. To this, foods were added in the order listed below during the 11 diet periods. The calcium content of the resulting diet is also shown.

*Feeding plan for entire study*

ADDITIONS TO BASAL DIET	LENGTH OF PERIOD	CALCIUM INTAKE mg/day
I. No additions	six 4-day tests	240
II. Milk, 400 gm	six 4-day tests	670
III. Cocoa, 5.6 gm; milk, 400 gm	five 4-day tests	679
Christmas vacation (15 days)		
IV. No additions	seven 4-day tests	226
V. Cocoa, 58.2 gm; milk, 420 gm	three 4-day tests	724
VI. Milk, 420 gm	four 4-day tests	684
VII. Cocoa, 28.5 gm; milk, 420 gm	five 4-day tests	699
VIII. Milk, 240 gm; vanilla ice cream, <sup>5</sup> 185 gm	six 4-day tests	755
IX. Cocoa, 34.8 gm; milk, 240 gm; chocolate ice cream, 185 gm	three 4-day tests	752
X. No additions	one 4-day and two 5-day tests	206
XI. Cocoa, 21.0 gm	one 4-day and two 5-day tests	226

(c) *Length of periods.* In the initial plan of the experiment, the intention was to have a minimum of five 4-day tests in each dietary period. Period V, which lasted only 12 days, was ab-

<sup>5</sup> Ice creams for the study were made at the University dairy with the same basic formula for both the chocolate and the non-chocolate. A single lot of each was used throughout the ice cream periods.

ruptly terminated because of the adverse effects of the large amount of cocoa. Period VI was shortened, therefore, since 4 of the subjects had already had 12 days on the milk diet.

After the first 12 days of period IX, it appeared that the amount of cocoa being fed in conjunction with the diet containing 650 to 750 mg calcium per day had not affected the calcium balances, and it was decided to institute a more rigorous test by use of a low-calcium diet. The time remaining was then equally divided between periods X and XI.

(d) *Reversal feeding.* All subjects did not follow the exact schedule above, due to a reversal system of feeding. This system was instituted in an attempt to minimize the effects of changes in metabolism with time or with uncontrolled environmental conditions. In brief, it consisted of dividing the subjects into two groups, 4 and 3 subjects in each, one of which followed the order listed above and the other the following order: I, III, II, IV, VI, V, VII, IX, XI, and X. In this fashion, when a diet comparison was to be made each of the two diets to be compared was being fed simultaneously to two groups. It was intended to have a reversal of period V, but, as explained above, the plan was abandoned when the three subjects consuming cocoa reacted unfavorably to the large amount.

### *Sampling methods*

(a) *Foods.* Aliquot portions of food were taken for analysis at the time of preparation. All foods were analyzed in 4-day pools. Bread and milk were each made into separate pools and the remaining foods composited. The food composites and milk pools were blended in a Waring Blendor and made up to a definite weight. They were preserved either with added acetic acid or by freezing until analyses were completed. Bread was prepared for sampling by drying at 110°C. and grinding.

(b) *Excreta.* Fecal periods were marked either by carmine or chromium oxide. The color of the carmine was difficult to detect in the feces of cocoa-fed subjects and the green color of the chromium oxide proved to be more easily detectable. Daily



urine samples and the fecal collections were composited into separate 4-day pools. The urines were preserved with either 1% hydrochloric acid or toluene. The fecal pools were blended with acetic acid and refrigerated until the analyses were completed.

### *Methods of assay*

Calcium and dry matter were analyzed according to the methods of the Association of Official Agricultural Chemists ('40), with a slight modification in the calcium method. In this instance, alizarin-S red was used in place of methyl red as an indicator. Nitrogen was assayed by the Kjeldahl method and gross energy determinations were made in the Parr oxygen bomb calorimeter.

### *Supplementary observations*

The physical well-being of the subjects was checked by daily weighings and by periodical physical examinations. Basal metabolism was determined by the gasometer technique (Boothby and Sandiford, '20) at the beginning of the study. Detailed records were made of unusual energy expenditures. No activity restrictions were invoked, however.

### *Estimation of calcium needs*

Calcium utilization and requirement values were calculated from average calcium balance data.

(a) Utilization values were calculated according to the method of Breiter et al. ('41) by the following formula:

$$\text{Per cent utilization} = \frac{\text{Net losses}^6 \text{ during basal period} - \text{net losses during test period}}{\text{Intake during test period} - \text{intake during basal period}} \times 100.$$

(b) Requirement values were determined according to the following formula (Outhouse et al., '41):

$$\text{Calcium requirement} = \text{Calcium intake} \pm \frac{\text{Calcium balance}}{\% \text{ utilization of calcium}} \times 100.$$

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<sup>6</sup>“Net losses” refers to the average daily calcium balance which, since the intake is below the estimated requirement, is negative, representing a loss.

## PRESENTATION OF DATA

The data from these experiments are presented in tables 1 to 3. Table 1 shows the average daily calcium balance data of each subject for the 11 dietary periods. Average values for all of the subjects appear in the last column. Calcium utilization and requirement data for each subject are shown in table 2. Table 3 reports the average daily fecal dry matter and nitrogen excretion of individual subjects for periods V through XI. Caloric content of the feces for the same periods (excluding VI and VII) are also shown.

## DISCUSSION AND RESULTS

The calcium balance data (see table 1) show considerable intra-individual variation. This was shown not only in the three periods (II, VI, and VIII) in which milk was added to the basal diet, but also in the three periods (I, IV, and X) in which the basal diet was fed alone. No consistent change in calcium balance was noted upon the inclusion of cocoa during any of the periods. The large amount of individual variation may account for the fact that no statistically significant difference was found between the average calcium balance observed on the cocoa and that on the non-cocoa diets. These averages for all subjects and all periods were  $-8.70 \pm 9.7^7$  mg for the cocoa diets and  $-12.85 \pm 11.0$  mg for the non-cocoa diets, the difference being insignificant due to the high standard errors.

The extreme amount of individual variation among subjects and between periods for the same subject led the authors to an investigation of the degree of difference required between average calcium balances to show a significant effect of a given cocoa diet on calcium utilization. Analysis of variance of the average calcium balance values showed that a difference of 29, 57, 36 and 23 mg for periods II and III, VI and VII, VIII and IX, and X and XI, respectively, would be required in order

<sup>7</sup> In this instance and in all other cases, the value defining the significance of the mean is the standard error.

to demonstrate a dietary effect with a probability of 0.05. Because of this high degree of variance, the data do not preclude the possibility that an effect of cocoa upon calcium utilization may have been obscured by uncontrolled factors.

In comparing the change in calcium balance from one period to the following period, it was noted that 6 of the 7 subjects showed a decreased calcium retention in going from period II to III. A similar decrease was noted in all 7 subjects from period VIII to IX. This similarity of response to diet change for all subjects without regard to order of cocoa feeding would indicate a probable effect of time of feeding. These data emphasize the importance of a reversal feeding technique since, in the above instance, if all subjects had been fed the same diet simultaneously and had proceeded as a group to the second diet, the observed change in direction of retention might have been attributed to a specific dietary effect.

The extent to which the oxalic acid content of the cocoa may have contributed to the variation in calcium balances is discussed in a preliminary report of this study (Mitchell and Smith, '45).

Additional evidence of the similarity in value of the calcium of the cocoa and non-cocoa diets is shown by the calculated utilization values in table 2. In computing these values, the calcium balance during the basal period was an average balance for the three low-calcium periods. All milk periods were averaged for the non-cocoa diet evaluation, and for the cocoa diet evaluation all periods in which cocoa and milk were fed were averaged, regardless of the amount of cocoa involved. According to these data on utilization, the calcium of the cocoa diets was as well utilized as was that of the non-cocoa diets, the average utilization for the former being 21.3% as compared with 20.0% for the latter. The difference was insignificant statistically. These values fall within the lower portion of the range of 15.3 to 35.1% for 7 adults reported by Breiter et al. ('41). Steggerda and Mitchell ('46), in a study of the calcium utilization of 19 adult men, report values ranging from 16 to 45%, with



Period VI			
Basal + 120 gm milk (four 4-day tests) Feb. 11-March 1, 1945			
Intake	689.3 <sup>1</sup>	681.2	700.8
Fecal excretion	510.7	508.2	604.1
Urinary excretion	187.0	165.5	210.4
Balance	-8.4	+7.5	-121.7
		+17.8	+17.8
			+3.9
			+60.5
			672.1 <sup>2</sup>
			684.0
			489.9
			197.5
			-3.4

Period VII			
Basal + 420 gm milk + 28.5 gm cocoa (five 4-day tests) March 3-22, 1945			
Intake	717.9 <sup>2</sup>	677.1	698.3
Fecal excretion	611.2	512.5	613.5
Urinary excretion	141.0	146.7	169.2
Balance	-34.3	+17.9	-84.4
		+16.7	+16.7
			+47.4
			-30.6
			+20.0
			702.2 <sup>2</sup>
			698.7
			530.2
			533.5
			172.0
			-6.8

Period VIII			
Basal + 240 gm milk, cookies, candy, and ice cream (six 4-day tests) March 24-April 16, 1945			
Intake	765.5 <sup>1</sup>	756.8	775.9
Fecal excretion	582.9	518.3	489.4
Urinary excretion	206.4	149.2	207.6
Balance	-23.8	+89.3	+78.9
		+89.3	+40.2
			-1.1
			+89.5
			+15.2
			719.0 <sup>1</sup>
			756.5
			533.9
			170.8
			+29.7

Period IX			
Basal + 240 gm milk + 34.8 gm cocoa in milk, cookies, candy and ice cream (three 4-day tests) April 18-29, 1945			
Intake	765.3 <sup>1</sup>	747.8	759.8
Fecal excretion	599.7	536.4	556.4
Urinary excretion	132.1	129.8	166.7
Balance	+33.5	+81.6	+36.7
		+81.6	+18.0
			+38.4
			+2.8
			741.4 <sup>2</sup>
			745.8
			513.6
			119.0
			165.8
			+40.5

Period X			
Basal low calcium diet (one 4-day and two 5-day tests) May 4-17, 1945			
Intake	248.4	181.9 <sup>1</sup>	215.7
Fecal excretion	214.4	148.2	151.3
Urinary excretion	117.4	125.3	149.5
Balance	-83.4	-91.6	-85.1
			-59.1
			-77.2
			-83.1
			-79.9
			191.5 <sup>1</sup>
			206.4
			159.7
			158.8
			126.6
			-79.9

Period XI			
Basal low calcium diet + 21.0 gm cocoa (one 4-day and two 5-day tests) May 18-31, 1945			
Intake	245.7	239.5 <sup>1</sup>	214.4
Fecal excretion	219.8	203.4	173.3
Urinary excretion	113.7	99.1	138.9
Balance	-87.8	-73.0	-97.8
			-35.2
			-57.6
			-44.2
			224.2 <sup>2</sup>
			235.7
			182.4
			109.2
			-65.9

<sup>1</sup>In the reversal feeding plan, these subjects consumed this diet during the time designated for the following period. Data were grouped thus for comparative purposes.

<sup>2</sup>Similarly, the data shown in this period were actually obtained at the time of the preceding period.

17 of the 19 values higher than any reported in the present study.

The average calcium requirement of the subjects when on the non-cocoa diet was  $669 \pm 42$  mg daily, as compared with  $686 \pm 52$  on the cocoa diet. Though 6 of the 8 subjects (see table 2) showed lower requirements when on the cocoa than on the non-cocoa diets, the differences for the entire cocoa and non-cocoa groups were not statistically significant. The overall average requirement of  $678 \pm 32$  mg daily agrees with the

TABLE 2

*Utilization of calcium from cocoa and non-cocoa diets, with individual calcium requirements calculated therefrom*

SUBJECTS	NON-COCOA DIET		COCOA DIET	
	Utilization	Requirement	Utilization	Requirement
	%	mg/day	%	mg/day
A	16.7	787	17.6	756
B	23.3	634	26.9	577
C	22.5	761	22.0	772
D	17.3	813	17.8	765
E	19.1	657	23.9	568
F	23.9	509	19.9	931
G	13.8	694	16.5	649
H	23.3	498	25.6	470
Average	20.0	669	21.3	686

findings of Outhouse et al. ('41), who reported an average requirement of 662 mg daily for 7 adults. The requirement of  $11.8 \pm 0.5$  mg/kg of body weight found in the present experiments is somewhat above the value of 10.7 mg/kg reported in the earlier investigation, the report of which also presents a comprehensive review of studies of calcium requirements published prior to 1941. Steggerda and Mitchell ('46), in the study mentioned above, indicate an average daily calcium requirement of 644 mg (S.D., 181), or 9.21 mg per kilogram body weight (S.D., 2.7) for the 19 men studied. Leverton and Marsh ('42) recommended a "minimal requirement" of 0.83 gm calcium per day for calcium equilibrium. This recommenda-

tion was made as the result of 100 calcium balance studies in college women on self-chosen diets, on the basis of the percentage of calcium retained at different levels of intake.

Although the calcium balances were not significantly altered when cocoa was removed from the diet, the division of calcium between urinary and fecal pathways was significantly affected. As may be seen in table 1, the fecal calcium fell and the urinary calcium rose with the removal of cocoa from the diet. Of the 27 cocoa comparisons made, 25 showed an increase in the urine calcium and 23 a decrease in the fecal calcium. Thus, the deviations from the ideal outcome of 13.5, if random factors alone operated, are 11.5 and 9.5, respectively. The standard deviation of 27 events which may occur in either one of two ways with equal probability is  $2.6 (S = \sqrt{27 \times .5 \times .5})$ . Thus, the deviations from the ideal outcome are 3.5 or more times the standard deviation, and can be considered highly significant.

The extent of the increase in fecal calcium, when results on cocoa diets were compared with those on non-cocoa diets, was not directly related to the 4 levels of cocoa ingested. These increases were  $26.7 \pm 11.7$ ,  $43.6 \pm 13.4$ ,  $29.4 \pm 14.4$  and  $22.7 \pm 10.6$  mg calcium daily when 5.6, 28.5, 34.8 and 21.0 gm cocoa were fed. In a similar comparison of decreases in urinary calcium, the values were  $13.5 \pm 5.9$ ,  $25.5 \pm 8.2$ ,  $43.4 \pm 8.3$  and  $17.3 \pm 4.3$  mg when the 4 above-mentioned levels of cocoa were fed. Using the "t" test of Student ('25), it was found that both the increases in fecal calcium and the decreases in urinary calcium were statistically significant.

There is no doubt that there was a distinct effect of the cocoa upon the route of calcium excretion when cocoa was added to diets comparable in all other components. Although its over-all effect did not appear to change the availability of dietary calcium, as evidenced by the lack of differences in the calcium balance data, there must be some property of cocoa which physiologically affects the route of excretion of calcium. Factors which may affect urinary calcium excretion have been summarized by Knapp ('47). Calcium intake, considered by

this author as a most important factor, could scarcely have explained the data in the present paper. The acid-base balance of the diet, another factor discussed, though changed somewhat with cocoa feeding, did not appear to be sufficiently altered to account for the urinary decrease. Ash from 100 gm cocoa was found to require 32 ml normal acid for neutralization. The decrease in urinary nitrogen and calcium accompanying the feeding of cocoa may be suggestive of some specific physiological effect of the latter.

A study of nitrogen excretion was undertaken after the largest amount of cocoa had been fed. When this amount of cocoa (56.2 gm) was fed to subjects B, C, and F in period V, headaches, nausea and decreased appetites were reported. The subjective symptoms were accompanied by more frequent and voluminous stools. The stools were all well-formed and gave no evidence of a diarrhetic effect of the diet. An investigation of the constituents of the feces was then undertaken in an attempt to determine the nature of the increase. This showed that the volume increase was accompanied by a similar increase in dry matter, which occurred in every case on inclusion of cocoa in the diet (see table 3). Analysis of variance showed that these increases in dry matter were not directly related to the amounts of cocoa fed.

The total gross energy value of the fecal excretion was also increased by cocoa feeding. This increase was in direct proportion to the dry matter excreted. No further analyses were made which would indicate the nature of the material which occasioned this increase in gross energy. The question arises as to whether undigested cocoa may have accounted for the change. With regard to caloric value, if none of the cocoa had been utilized, the caloric increase in the feces would have been twice that observed. When the gross energy per gram of fecal organic matter (dry matter—ash) was calculated, it was found that this value was lower on the cocoa than on the non-cocoa diet in 12 of 16 comparisons. These data suggest that the increased gross energy of the feces could not have been due to impaired fat digestibility.



	Fecal dry matter (gm)					
Period V	22.1	38.0	47.2	22.1	17.6	15.3
Milk diet				...	...	...
Milk diet + 58.2 gm cocoa						
Periods VI and VII						
Milk diet	19.8	15.8	29.1	20.9	18.2	16.8
Milk diet + 28.5 gm cocoa	36.6	27.4	40.6	32.6	28.6	29.0
Periods VIII and IX						
Milk diet	20.6	14.7	25.4	21.3	19.1	16.0
Milk diet + 34.8 gm cocoa	32.2	27.9	36.8	29.9	27.1	23.1
Periods X and XI						
Low calcium diet	17.0	12.5		14.9	15.2	15.0
Low calcium diet + 21.0 gm cocoa	28.0	22.2		26.1	21.7	18.7
				Caloric value of feces		
Period V	124.3	193.3	247.4	111.9	96.7	71.8
Milk diet						
Milk diet + 58.2 gm cocoa						
Periods VI and VII						
Milk diet	113.2	80.9	167.4	114.5	105.7	87.3
Milk diet + 28.5 gm cocoa	198.1	143.2	234.1	164.3	148.4	149.9
Periods X and XI						
Low calcium diet	105.6	76.5		88.8	88.0	82.1
Low calcium diet + 21.0 gm cocoa	168.2	137.1		147.6	119.9	111.4
				Fecal nitrogen (gm)		
Period V	1.14	2.11	3.41	1.04	1.02	0.82
Milk diet				...	...	...
Milk diet + 58.2 gm cocoa						
Periods VI and VII						
Milk diet	0.99	0.66	1.86	1.00	0.94	0.84
Milk diet + 28.5 gm cocoa	2.19	1.52	2.80	1.95	1.73	1.75
Periods VIII and IX						
Milk diet	0.90	0.61	1.52	1.10	0.88	0.72
Milk diet + 34.8 gm cocoa	1.94	1.62	2.61	2.04	1.81	1.39
Periods X and XI						
Low calcium diet	0.86	0.68	...	0.77	0.83	0.61
Low calcium diet + 21.0 gm cocoa	1.36	1.52	...	1.47	1.05	1.47

There was also an increase in fecal nitrogen with cocoa feeding (see table 3). This increase was no greater than the amount of cocoa nitrogen consumed, in 13 of 14 cases. In fact, additional fecal nitrogen amounted, on the average, to 84% of the extra nitrogen ingested on the cocoa diet. The increased fecal nitrogen shows a poorer digestibility of the protein in the diet when cocoa is included. A depression in the digestibility of protein has been noted by other investigators when cocoa was included in the diet. Cohn (1895) reported that 52.7% of the nitrogen remained undigested when cocoa was included in the mixed diet of human subjects. Neumann ('06), in studies with one subject, reported a decrease in the apparent digestibility of protein from 82.5% on a basic diet of bread, cheese and sausage to 75% on the same diet with 35 gm cocoa added. He attributed the loss of nitrogen to the increased fecal material caused by the cocoa, since he observed that the fecal nitrogen rose and fell with the amount of dry feces. Pincussohn ('07) also noted an unfavorable use of nitrogen by women consuming cocoa in a mixed diet. Lipman and Mueller ('41) reported a study on rats in which a decrease in the apparent digestibility of milk proteins from 85.3% to 79.3% occurred when 15.8% of American process cocoa was included in the diet. Mitchell et al. ('26) reported a true digestibility of 38% for cocoa nitrogen when fed to rats as the only source of nitrogen in the diet.

The increase in fecal nitrogen was accompanied by a decrease in urinary nitrogen so great that larger positive balances were observed with cocoa additions in 13 of 16 possible comparisons. The improved balances during the cocoa feeding were in most instances no greater than the increased nitrogen intake incident to cocoa feeding (two exceptions). In all instances where nitrogen balances were determined, they were positive whether cocoa was included or not. Neumann ('06), in the study cited above, also observed a decrease in urinary nitrogen along with an increase in fecal nitrogen. It is interesting to speculate as to whether the theobromine content of the cocoa may have accounted for the lowered urinary excre-

tion of nitrogen. Bernheim and Bernheim ('46) have shown that 0.3 gm of theobromine, given with each meal to two women who were not habitual drinkers of tea or coffee, caused a consistent decrease in the excretion of urinary nitrogen after the third day of administration. This same effect was noted in rabbits injected with doses of theobromine, although a tolerance gradually developed. Quantities of theobromine not unlike those used by the above workers were involved in the present study. The analyzed theobromine content of the cocoa was 1.79%; thus, when 56.2 gm of cocoa were fed, as in period V, the subjects were receiving 1.0 gm theobromine daily. Bernheim and Bernheim suggest an inhibition of urea formation in the liver as a cause of lessened urinary nitrogen, since they found that the total urinary nitrogen was accompanied by a decrease in urea nitrogen, with no consistent variation in the concentration of the other nitrogen compounds of the urine and decreases in urea and non-protein nitrogen of the blood. The nitrogen retention reported by Benedict ('16) after caffeine administration to one man suggests that this compound may also have exerted its effect by interference with urea formation.

#### SUMMARY AND CONCLUSIONS

The effect of the ingestion of a moderate-cost American process cocoa on calcium utilization was studied in 8 college women, 18 to 22 years of age. This involved estimation of calcium balances for 208 consecutive days, except for 15 days intervening for the Christmas holidays. Five levels of cocoa (5.6, 21.0, 28.5, 34.8 and 56.2 gm) were tested, 4 in diets containing milk as the major source of calcium and the other (21.0 gm) in a milk-free diet. Because of the bulky nature of the stools on the cocoa periods, additional observations were made on stool composition.

A summary of the results follows:

1. No statistically significant differences were demonstrated between the calcium balances of subjects on non-cocoa diets and those of the same subjects on cocoa diets. For all periods

and subjects the daily calcium balances averaged  $-12.85 \pm 11.0$  and  $-8.70 \pm 9.7$  mg for the non-cocoa and cocoa diets, respectively.

2. This similarity of response is further illustrated by the calculated calcium utilization values. For the 8 subjects, the latter values averaged  $20.0 \pm 1.3\%$  on the non-cocoa diets and  $21.3 \pm 1.4\%$  on the cocoa diets.

3. The average daily calcium requirements for all subjects were  $686 \pm 52$  mg on cocoa and  $669 \pm 42$  mg on non-cocoa diets. The average requirement for all subjects on all diets was then  $678 \pm 32$  mg daily, or  $11.8 \pm 0.5$  mg per kilogram of body weight.

4. Even though significant changes in calcium balance were not demonstrated to result from the addition of cocoa to the diet, there was a significant effect upon the path of calcium excretion. With the inclusion of cocoa, in 25 of 27 comparisons the urinary calcium fell, and in 23 of 27 cases the fecal calcium rose above that of adjacent periods in which cocoa was omitted.

5. With cocoa feeding, increases in the fecal excretion of dry matter and nitrogen were observed in each of 4 dietary comparisons. Total calories excreted, increased in each of three possible comparisons.

It may be concluded that the tolerance of the subjects of this experiment for cocoa of the grade used was approximately one ounce daily, and that any possible deleterious effect of cocoa on calcium utilization was completely obscured by the variation (both within groups and within subjects) of the experimental data.

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# THE EFFECT OF STORAGE ON THE NUTRITIONAL QUALITIES OF THE PROTEINS OF WHEAT, CORN AND SOYBEANS

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Grains are stored for various periods of time before consumption by farm animals or man. When there is a heavy carry-over from one crop year to another, or when the government attempts to stabilize grain prices by encouraging the storage of surplus grain, the storage periods may extend over many months. During such storage, changes in the chemical composition and nutritive value of the grain may occur. The nature and extent of these changes will depend upon the initial condition of the grain, particularly upon its moisture content, and upon the conditions of storage. Reviews of the literature relating to the chemical and nutritional changes occurring in grain during storage have been published by Jones and others ('43), Watson ('46), and Zeleny ('48).

Jones and co-workers have observed changes in the chemical properties of the proteins of corn (Jones, Divine and Gersdorff, '42), of wheat and wheat flour (Jones and Gersdorff, '41), and of soybeans (Jones and Gersdorff, '38), revealed by a decrease in solubility and a partial hydrolysis yielding free amino nitrogen. In all cases, digestion studies *in vitro* indicated a decreased digestibility of the proteins, and ad libitum feeding trials with stored corn revealed a diminished weight gain per gram of protein consumed, a change that might have resulted from a decrease in the palatability of stored, as compared with fresh, corn. The extent of the observed changes

depended upon the temperature and duration of storage, the type of container, and the nature of the material stored. Ground grains were less stable in storage than whole kernels. In the experience of Pickett ('48), the proteins of the peanut are quite stable during long periods of storage. Practical feeding trials with chickens have failed to indicate deterioration in the proteins of fish meals after short storage (Evans, Carver and Hamm, '44), while similar trials with pigs detected no deterioration in wheat stored for 4 years (Longwell, '45).

It may be noted that the corn and wheat samples studied by Jones and associates ranged in moisture content from 10 to 15%, on the borderline level, or below, for the inhibition of mold deterioration, according to Snow, Crichton and Wright ('44) and Milner and associates ('46, '47). Throughout the storage period of two years used by these workers, all samples remained free from any indication of insect infestation or mold infection.

More precise studies of the nutritional deterioration of food proteins during storage were reported by Henry and Kon ('48) using spray-dried skim milk of low moisture content, 3 to 7%, stored at room temperatures or higher. In rat metabolism studies, protein digestibility was slightly, if at all, impaired, except with powders containing 5% or more of moisture, but the biological value of the protein dropped from 86.9 to 65.9 during a storage period of 85 days at 37°C. The deterioration in protein quality seemed to relate almost entirely to lysine, since lysine supplementation corrected the damage. This is but another illustration of the sensitivity of milk proteins to environmental factors, shown by Fairbanks and Mitchell ('35) to obtain following brief exposures to high temperatures, as in spray drying. In this case, however, cystine was the point of attack. The changes in the essential amino acid content of the proteins of dry skim milk resulting from storage have been reported by Hodson and Krueger ('47), and by Henry, Kon, Lea and White ('48).



The experiments reported in this article were designed to study the effect on the utilization by growing rats of the proteins of wheat, corn and soybeans of storage for periods up to almost three years, under conditions that would favor continued respiration and enzyme action but would inhibit insect infestation and mold infection. The effects on the protein quality of corn produced by specific fungi in the field have been investigated by Mitchell and Beadles ('40) and by Mitchell, Beadles, Koehler and Dungan ('47). It will be shown that, under the chosen conditions, the proteins of corn and of wheat are resistant during storage to changes that would impair either their digestibility or their biological value. On the other hand, the proteins of the soybean undergo a considerable depression not only in digestibility but also in biological value.

#### EXPERIMENTAL PROCEDURES

The grains were all of the 1942 crop and the samples used in the experiment were obtained from the Department of Agronomy through the courtesy of Dr. George H. Dungan. They were fumigated before storage and were analyzed for proximate constituents, with the results shown in table 1.

Each of the grain samples was stored in two conditions, as whole kernels and as a meal ground to pass through a 1 mm sieve. The ground soybean sample was defatted and autoclaved for 90 minutes at 17 pounds steam pressure, to simulate commercial expeller-processed meal. The samples were stored in screw-top tin cans, capacity about one gallon, and were sealed with paraffin. Each container held enough sample for one biological assay with rats of the digestibility and biological value of protein. The cans, filled with whole or ground grains, were stored in a temperature-controlled room for periods up to about three years. Cans containing whole and ground samples of the same grain were withdrawn simultaneously and periodically for assay. The room temperature was maintained at about 78°F. or higher during extremely hot weather.

The experimental rations for the protein metabolism studies on growing rats contained salt mixture 446 (Spector, '48) 4%,

TABLE 1  
*Chemical description of samples*

SAMPLE	DATE RECEIVED	DATE STOR'D	DATE OF FIRST TEST	PERCENTAGE COMPOSITION OF SAMPLES					
				Moisture	Protein (N x 6.25)	Ether extract	Ash	Crude fiber	Nitrogen- free extract
Wheat, Turkey Red variety	10- 2-42	10- 5-42	10-28-42	12.00	13.69	1.44	1.90	2.10	78.87
Soybeans, Illini variety	2- 9-43	3- 8-43	3-19-43	6.63 <sup>1</sup>	32.88	20.80	4.92	5.42	29.35
Corn, Station Yellow Dent no. 1	4-11-43	4-26-43	5- 6-43	9.23	9.88	4.47	1.27	1.91	73.24
Corn, U. S. Hybrid 13 <sup>2</sup>	5-11-43	6-21-43	6-12-43	11.56	8.19	4.30	1.23	1.94	72.78

<sup>1</sup> After defatting, autoclaving and dehydrating, these soybeans contained 6.17% moisture.

<sup>2</sup> (WF9  $\times$  38-11) (Hy  $\times$  L317).

NaCl 1%, fiber (wood flock)<sup>1</sup> 2%, wheat germ oil 0.5%, cod liver oil 1.5%, the test grain to the desired protein percentage, lard to 12% of total fat, sucrose 10% or less, and starch, or starch and glucose 1:1, to make up to 100%. The rations containing soybeans and wheat were made up to contain 10 to 12% protein ( $N \times 6.25$ ), but the corn rations could not be made to contain more than 7 to 8% protein. The soybeans that were stored as whole kernels before incorporation into the experimental rations were defatted, autoclaved and dried in the same manner as the soybeans that were stored in a ground condition.

The digestibility and biological value of the test proteins were determined by the nitrogen metabolism method developed in this laboratory (Mitchell, '24a; Mitchell and Carman, '26), with some later modifications. These modifications include the use of a feces marker ( $Fe_2O_3$ ); a reversal system of feeding whereby each rat receives samples of grain stored both as whole kernels and as a ground meal, in different experimental periods, 5 rats in one order and the other 5 in the reverse order; and a system of paired feeding which involves treating the 10 rats as 5 pairs for which the food intake is equalized in all periods. In each experiment, a sample of ground, dried and defatted beef round was tested along with the grains, generally by the same rats though at times in a separate test carried out simultaneously.

In the course of the two- to three-year experiment, several samples of beef were prepared and used, and all beef samples were stored in a sealed jar at 0°F. In each experiment, the determination of the metabolic fecal nitrogen per gram of food and of the minimum endogenous urinary nitrogen per  $W_{gm}^2$  was made in a single centrally located period, or in an initial and a final period. All rations used in a single experiment were equalized in nitrogen, fiber and fat content and each experiment involved 10 to 15 weanling albino rats. The pre-

<sup>1</sup> Purchased from Brown Co., of Portland, Maine. Contains 94.3% cellulose, a trace of lignin and only 0.0033% nitrogen.

liminary periods in all experiments were from 4 to 7 days in length and the collection periods, 7 days.

The samples of soybeans, stored for 34 months, either whole or after grinding, defatting, and autoclaving, were tested for digestibility and the biological value of nitrogen, alone and when supplemented with methionine.

#### EXPERIMENTAL RESULTS

The average experimental results are summarized in table 2, including: (a) the digestibility of the protein with due allowance for the metabolic fecal nitrogen; (b) the biological value, representing the percentage of the absorbed nitrogen retained for maintenance (replacement of endogenous losses) and for growth; and (c) the net utilization, obtained by multiplying the digestion coefficients by the biological values and dividing by 100.

The standard errors of these average computations have not been given, because they are not necessarily valid in the comparison of samples taken at different times, and because they are of little use in the comparison of whole and ground grains sampled at the same time. The latter comparisons are best carried out according to the Student ('25) method of paired differences, since in each test grains stored whole and ground were generally fed to each of the test animals.

The pooled standard deviation of all individual coefficients of digestion,<sup>2</sup> computed from the sum of the squared deviations of individual coefficients about their respective group means, is 2.76. The corresponding standard error for the mean of a group of 10 is 0.87. The standard deviation of individual biological values, similarly computed, is 4.49, equivalent to a standard error of 1.4 for the mean of a group of 10. These latter two values may be compared with those reported earlier

<sup>2</sup>Omitting the coefficients of digestion for beef, since the variation of such coefficients is artificially minimized by the practice of considering all estimated coefficients above 100 as being equal to 100. The standard deviation of individual digestion coefficients of 2.76 is probably higher than what might be called normal because of the rather frequent occurrence of diarrhea in the experimental rats, especially in tests 334, 340, 344 and 346.

TABLE 2

*Average results of the biological tests<sup>1</sup>*

TEST NO.	STORAGE PERIOD	STORED AS WHOLE KERNELS				STORED IN GROUND CONDITION				BLEF ROUND STANDARD			
		True digestibility	Bio-logical value	Net <sup>2</sup> utilization	%	True digestibility	Bio-logical value	Net <sup>2</sup> utilization	%	True digestibility	Bio-logical value	Net <sup>2</sup> utilization	%
	days	%	%	%	%	%	%	%	%	%	%	%	%
318	0	88.80	49.1	43.6		Wheat							
						88.80	49.1	43.6		90.80	72.4	72.3	
331	354	89.18	53.5	45.5		85.88	50.6	43.5		98.25	70.9	75.7	
348	730	88.40	46.2	40.8		89.80	46.5	41.8		99.50	79.2	78.8	
325	0	85.30	69.1	58.9		Soybeans							
						85.30	69.1	57.3		99.30	73.3	72.8	
334	256	78.30	62.6	49.0		85.70	73.7	63.2		99.44	78.6	78.1	
344	404	80.00	66.1	52.9		84.67	69.2	58.6		98.70	75.3	74.3	
371	1020	75.00	56.0	42.1		82.80	64.0	53.0		99.60	78.3	78.0	
326	0	92.60	55.2	51.1		Corn, Station Yellow Dent							
						92.60	55.2	51.1		99.50	78.2	77.8	
337	245	93.00	52.9	49.2		93.80	53.4	50.1		99.80	83.6	83.4	
346	417	92.10	50.6	46.6		92.80	51.5	47.8		99.70	73.6	72.9	
372	958	91.00	56.4	51.3		92.20	55.2	50.9		99.90	80.7	80.6	
329	0	91.40	64.9	59.3		Corn, U. S. Hybrid 13							
						91.40	64.9	59.3		99.70	81.6	81.4	
340	244	90.90	57.7	52.5		90.70	58.6	53.2		98.60	82.6	81.4	
369	850	90.17	60.2	54.4		91.50	60.6	55.4		99.92	82.8	82.8	

<sup>1</sup> With few exceptions, each value in this table is the average of values for 10 rats. In one experiment (331) the values given are averages for 8 rats, and in another (369) they are averages for 12 rats.

<sup>2</sup> Percentage digestibility multiplied by percentage biological value and divided by 100.

from this laboratory by Mitchell, Burroughs and Beadles ('36) of 3.7 and 1.2, respectively. The standard deviation of individual net utilization values, computed by the same method, is 4.44, equivalent to a standard error of 1.4 for the mean of a group of 10.

#### DISCUSSION

##### *Changes in wheat and corn*

The interpretation of the data in table 2 with reference to the main problem of the study, i.e., the effect of the storage of grains on the nutritive value of their proteins, should not be carried out on the basis of the intra-group standard deviations and the group mean standard errors, because these do not necessarily apply to inter-group variations when tests are conducted with different consignments of rats at widely different times. In assessing inter-group variation on diets similarly constituted, it was hoped that the results secured with the beef preparations, tested 14 times and at widely different intervals, would serve the purpose. However, as was explained above, the protein contents of the meat diets varied from 7.50 to 12.00 in accordance with the protein contents of the grain diets with which they were simultaneously tested. While this variation probably exerted no effect on the coefficients of digestibility, it would presumably modify the biological values. According to previous information secured in this laboratory (Mitchell, '24a; Mitchell and Beadles, '27), the relationship between biological values and the protein content of the diet is an inverse one. In the present case, the regression of biological value on protein content of diet is not linear, but of the following type:  $\frac{x}{y} = -0.021 + 0.015x$ , in which  $x$  is the percentage of protein ( $N \times 6.25$ ) in the diet and  $y$  the associated biological value. The standard deviation of the observed mean biological values about the above regression line (i.e., the square root of the sum of squared deviations of observed from calculated  $y$ 's divided by the number of degrees of freedom) is 3.02 (see table 3).

The standard deviation of the biological values of beef protein about the regression line relating biological values to

dietary protein levels, 3.02, may be used in detecting time changes in the biological values of the proteins of stored grains. An inspection of the biological values in table 2 suggests that, for stored wheat and stored corn, no time changes occurred. Such variations as occurred from one sampling to another were irregular; in particular, they reveal no consistent downward trend with lengthening time of storage, and for the Station Yellow Dent corn the final values are the same, or

TABLE 3

*The relation between protein content of diet ( $x$ ) and biological value of the protein of beef ( $y$ ) as described by the equation:  $\frac{x}{y} = -0.021 + 0.015x$*

EXPERIMENT NUMBER	PROTEIN IN DIET	BIOLOGICAL VALUES		DIFFERENCES
		Observed	Calculated	
	%	%	%	%
331	12.00	76.9	75.5	— 1.4
348	11.88	79.2	75.6	— 3.6
318	11.62	72.4	75.8	+ 3.4
334	10.81	78.6	76.6	— 2.0
325	9.94	74.5	77.6	+ 3.1
371	9.94	78.3	77.6	— 0.7
344	9.69	75.3	77.9	+ 4.6
346	8.62	73.6	79.6	+ 6.0
337	8.50	83.6	79.8	— 3.8
372	8.38	80.6	80.0	— 0.6
326	8.31	78.2	80.2	+ 2.0
369	7.69	82.8	81.5	— 1.3
340	7.59	82.6	81.8	— 0.8
329	7.50	81.6	82.0	+ 0.4

somewhat higher, than the initial values. This impression is confirmed by the value for the pooled standard deviation of all mean biological values for corn and wheat samples about their respective sample means, i.e., 3.05. This standard deviation is almost identical with the standard deviation of mean biological values for the same protein mixture, that of beef, tested at different times through a period of two to three years, i.e., 3.02.

The variation to be expected from uncontrolled factors in the experiment affecting the digestibility of proteins cannot be assessed from the 14 mean coefficients of digestibility of

beef protein carried out at different times, because the digestibility of this protein ( $N \times 6.25$ ) is so near 100 and because individual coefficients corrected for metabolic fecal nitrogen were entered as 100 whenever the estimation indicated a value over 100. Hence, resort must be had to the pooled standard deviation of individual values measured simultaneously about their respective group means.

The variation in average digestibility of the protein fraction of stored wheat and corn samples neither reveals nor suggests a depression of digestibility with lengthening time of storage. The standard error of a mean coefficient for a group of 10 rats tested simultaneously is 0.87, and that of a difference between the means of two such groups is 1.23. For an observed average depression in digestibility to be significant at the 2% level, assuming an improved digestibility on storage to be inconceivable in view of prior work indicating a reduction in protein solubility as storage progresses (Jones and Gersdorff, '41; Jones, Divine and Gersdorff, '42), the difference between means would need to exceed 2.47. Of the differences in mean digestibility of the protein for corn and wheat within any one series of tests, only two exceed 2.47, i.e., in the comparison of wheat, stored in the ground condition, in experiments 318 and 331, and 348 and 331. For these comparisons the variation in protein digestibility is irregular, with the highest coefficient obtained with the sample stored the longest.

The probable insignificance of all differences in mean digestibility from one sample of grain to another stored for a longer period is further indicated by the fact that the pooled standard deviation of the 20 mean digestion coefficients for corn and wheat samples about their respective group means,  $1.04 \pm 0.20$ , is not to be distinguished statistically from the standard error of a mean coefficient, 0.87, estimated from the variation among individual coefficients secured simultaneously.

The net utilization values for beef protein tested at 14 different times are correlated inversely with the protein content of the diet, as were the biological values, and for the same reason. In this case, the regression equation is  $\frac{x}{y} = -0.022$



+ 0.015x, in which x is the percentage protein in the diet and y is the average net utilization of beef protein. The standard deviation of mean net utilization values about this regression line is 3.10. The standard error of a difference between two mean values would be 4.3. A difference between two means of 10 items each must be as large or larger than  $4.3 \times 1.88 = 8.1$  to possess significance at the 2% level of probability. None of the differences between mean net utilizations for the same grain sample tested at different stages of storage is of this magnitude. For only the U. S. Hybrid corn sample is there any suspicion that storage may affect protein utilization, and here the difference between the values for the initial sample

TABLE 4

*Differences in the mean digestibility and biological values of corn and wheat stored as kernels or as ground meal<sup>1</sup>*

	GRAINS STORED WHOLE	GRAINS STORED GROUND	DIFFERENCES
Digestibility	90.25 $\pm$ 0.54	90.85 $\pm$ 0.22	0.60 $\pm$ 0.59
Biological value	53.71 $\pm$ 0.39	53.99 $\pm$ 0.26	0.28 $\pm$ 0.47
Net utilization	48.56 $\pm$ 0.45	49.10 $\pm$ 0.27	0.54 $\pm$ 0.52

<sup>1</sup> The means are given with their standard errors and were obtained by pooling the results of 7 samplings of stored wheat and corn.

and for the final samples after 850 days of storage is 4.9 percentage units for the samples stored whole, and 3.9 percentage units for the samples stored as a ground meal.

It is quite commonly supposed that grains keep better when stored as whole kernels than when stored as ground meals. For the corn and wheat samples studied in this investigation, and with reference to protein utilization by growing rats, this was not in general true. The statistics given in table 4 were secured by comparing whole and ground grains without reference to pairing. The differences between stored whole grains and stored ground meals in mean digestibility, biological value and net utilization of nitrogen are evidently insignificant statistically.

Paired comparisons were made in those experiments in which the mean digestion coefficients or biological values differed to such an extent as to suggest a difference between kernel samples and ground samples, i.e., in experiments 337, 346, 348, 369 and 372. In these comparisons, the differences in digestibilities or biological values for each rat on stored whole sample and on stored ground sample were analyzed by the Student ('25) method. None of the differences proved to be highly significant ( $P = 0.02$  or less), though one of them approached significance. In experiment 348, involving wheat samples stored for 730 days, the mean digestibility of nitrogen in the whole grain storage sample was 88.40, and that in the ground grain storage sample was 89.80%. The "t" value of the difference is 2.28 and the probability that a random combination of the uncontrolled factors in the experiment would have produced a difference, in the same direction, as great or greater than this, is 0.024. It is to be noted that, in this case, the nitrogen of the ground sample exhibited the higher average digestibility. The other probabilities obtained in these paired comparisons ranged from 0.14 to 0.29.

It is worthy of note that the biological values for whole wheat protein recorded in table 2, averaging 49.2, are less than the average of 67 previously reported from this laboratory (Mitchell and Carman, '24) for wheat of unrecorded variety. In unpublished experiments we have since found a biological value of 43.4 for the proteins of whole wheat flour from durum wheat. There is more than a suspicion on the basis of these findings that there are marked differences among different varieties of wheat in the nutritional value of their proteins. The proteins of the open-pollinated variety of corn exhibited an average biological value of 53.6 and those for the hybrid variety, 61.1. These values agree well with the value of 60 previously reported for corn (Mitchell, '24b).

### *Changes in soybeans*

The soybeans stored in a ground condition, preheated and defatted, varied in protein digestibility and biological value

from sample to sample in an irregular fashion. The changes in protein utilization were too small to be considered significant, except for the changes that occurred from 404 days of storage to 1,020 days. In this interval, the protein digestibility dropped from 84.67 to 82.80, a drop of 1.87; the biological value dropped from 69.2 to 64.0, a difference of 5.2; and the net utilization from 58.6 to 53.0, a decrease of 5.6. Judged by the criteria used in the interpretation of the values secured with the wheat and corn samples, these differences are suggestive of storage effects but do not constitute conclusive evidence. The drop in digestibility of 1.87 is less than the critical difference of 2.47 between means of 10 variates. The drop in biological value of 5.2 is only slightly more than the standard error of a difference between the means of two groups of 10 variates sampled at different times, 4.3 [ $\sqrt{(3.02)^2 + (3.02)^2} = 4.3$ ]; while the drop of 5.6 in net utilization is much less than twice the standard error of a difference between two means of 10 variates each, i.e., 4.3.

The soybeans stored whole, however, tell a different story. For 404 days of storage, the changes in biological value in these samples were irregular and not clearly indicative of an effect of storage. The fall in digestibility during this period from 85.30 to 80.00 is clearly a real change if judged by the critical value of 2.76 used in the analysis of the wheat and corn data. While this value is based upon intra-group variation, for reasons given above, it seems to be fairly applicable to the variation of means of samples taken at different times.

Between 404 and 1,020 days of storage the average digestibility of the soybeans stored whole dropped 5.00 percentage units, the mean biological value dropped 10.1 percentage units, and the mean net utilization dropped 10.8 percentage units. The reality of these decreases in protein utilization seems clear by the criteria of significance developed in the preceding section.

The sample of soybeans stored in the unground condition may be readily compared with respect to utilization of protein with the sample preheated and stored in the ground condition,

since in each of the three tests each experimental rat received both types of bean samples. In protein digestibility, the soybeans preheated and stored as a defatted meal exceeded the soybeans stored whole and unheated by 7.40 percentage units at 256 days of storage, by 4.67 percentage units at 404 days of storage, and by 7.80 percentage units at 1,020 days. In each case all paired comparisons favored the beans heated before storage, so the statistical significance of the differences is clearly established.

A similar comparison of biological values favored the samples heated prior to storage, the average differences being 11.1, 3.1 and 8.0 percentage units at 256, 404 and 1,020 days of storage, respectively. Statistical analysis of the individual paired data showed the first difference to be highly significant, data from all rats agreeing in the verdict. The biological values in the second test (404 days of storage) were so variable that no significance can be attached to the average difference. At 1,020 days' storage, the difference in biological value was significant at the 5% level ( $P = 0.050$ ).

The whole picture supports strongly the conclusion that soybeans stored at 75° to 80°F. without pretreatment undergo changes in chemical composition that induce a marked reduction in the digestibility of their nitrogen and in its biological value for the growing rat. These changes are not brought about by insect infestation or by any observed mold infection, and they occur in beans containing less than 10% of moisture. Under the conditions of this investigation, the impairment in nitrogen digestibility averaged 10 percentage units in 1,020 days of storage, and the impairment in biological value averaged 13 percentage units. The total impairment in the nutritive value of nitrogen, as measured by the net utilization values, amounted to about 17 percentage units, representing a decrease of 28%. This depression in the nutritive value of the proteins of soybeans on storage is largely prevented by pretreatment with heat, 90 minutes at 17 pounds steam pressure. The grinding and defatting of the beans prior to autoclaving presumably were minor factors, if they operated at all, in these changes.

The conclusion that grinding prior to storage is a minor factor in protein preservation under conditions that eliminate the action of insects and molds is based upon the information secured with corn and wheat.

Soybean proteins are deficient nutritionally in the sulfur-containing amino acids. This fact has been demonstrated by Mitchell and Smuts ('32), Shrewsbury and Bratzler ('33), and many others. This is also true of the heated soybean (Mitchell, Hamilton and Beadles, '45). The effect of supplementing with methionine the protein in the final samples of soybeans, stored either whole or ground but in both cases autoclaved before testing, was measured by the nitrogen balance technic previously described. The average results for this test are summarized in table 5.

From these data it is evident that methionine supplementation had no demonstrated effect upon nitrogen digestibility in either sample of soybeans, but that it exerted a definite effect in raising the biological value of the nitrogen in each sample. The favorable effect of methionine supplementation was much greater for the beans heated before storage than for those not so treated. In the first case, the average biological value was increased by 20.2 percentage units, from 58.3 to 78.5; in the case of the beans stored whole, the biological value was raised only 6.0 percentage units, from 54.0 to 60.0. Both increases were highly significant statistically.

Since methionine supplementation improved the biological value of the proteins in both storage samples of soybeans, and since methionine (or cystine) effectively supplements soybeans, raw or heated, that have not been stored (Mitchell, Hamilton and Beadles, '45), it follows that the damage inflicted by storage on the metabolic utilization of soybeans is due primarily to an effect upon the cystine or methionine (or both) contained in the soybean protein. The fact that methionine supplementation improved the biological value of the pre-heated soybeans, after storage, much more than that of the

untreated soybeans stored as whole beans, indicates that the greater storage damage in the latter case involved also other amino acids than cystine and methionine.

TABLE 5

*Average results of supplementing with methionine the proteins of soybeans stored ground and as whole kernels for 34 months. (Each value relates to a group of 6 growing rats)*

	TRUE DIGESTI- BILITY	BIO- LOGICAL VALUE
	%	%
I. Soybeans stored as a defatted autoclaved meal for 34 months		
No methionine supplement	83.8	58.3
With methionine supplement	85.7	78.5
mean differences	+ 1.8	+ 20.2
standard deviation	3.97	8.73
probability	0.18	0.0017
II. Soybeans stored as whole kernels for 34 months		
No methionine supplement	77.2	54.0
With methionine supplement	76.8	60.0
mean differences	- 0.33	+ 6.0
standard deviation	2.16	2.10
probability	0.37	ca. 0.0005

### *Interpretations*

The results of the experiments on corn and wheat, indicating a high resistance of cereal proteins to nutritional change by agents that operate during storage at a low moisture level, are in agreement with the findings of Robertson, Lute and Gardner ('39) that cereals (wheat, oats and barley) stored at a moisture level of 10% may be expected to last for two years or more if in good condition initially. In fact, they may retain 80% of their original ability to germinate after 15 years' storage (Robertson and Lute, '37). As Oxley ('48) has pointed out, ". . . germination is the most sensitive characteristic of grain and hence it is generally true to say that if the germination is good, the grain is completely undamaged from the point of view of milling."

In another connection Oxley says, "Cereal grains are seeds, that is, they are the dormant resting stage of the plant which bears them. Though they are alive, and all living things are continually respiring, producing heat, water, and carbon dioxide, they are at a very low ebb. It is the combined effect of their continued life (which enables them to resist decomposition by micro-organisms) and the very low level of that life, which makes cereal grains such pre-eminently stable bodies in store."

The results obtained with cereal grains are not necessarily opposed to the findings of Jones and co-workers ('41, '42), since the changes in solubility of the proteins in saline solutions and the partial proteolysis observed by these workers in stored grains do not necessarily imply a change in the nutritive value of the proteins. The decreased digestibility noted in tests carried out *in vitro* refers to rate of digestion rather than to the completeness of digestion, while the decreased growth-promoting power reported for the proteins of stored corn in ad libitum feeding trials may have been brought about solely by a diminished food consumption conditioned by an impairment in the palatability of the corn during storage.

The marked detrimental effect of prolonged storage on the nutritive properties of the soybean, when the bean is not subjected to pre-treatment other than fumigation, places the soybean in marked contrast to the cereal grains. The possible causes for this distinction may rest largely in the difference in anatomical structure. The living part of the seed is the embryo or germ. The embryo accounts for only 2% or less of the wheat kernel and 9 to 10% of the corn kernel, but in the soybean it accounts for 92% of the bean, which consists entirely of seed coat and embryo. Embryonic respiration would be expected to be more intense in the soybean, therefore, than in corn or wheat seed. The soybean contains a wide variety of enzymes (Waksman and Davison, '26), some of which may attack the amino acids produced from the seed proteins through protease action. The fact that preheating of soybeans largely eliminates the detrimental effect of storage

on the nutritional quality of the proteins is in harmony with the view that the reactions involved are to a large extent enzymic in nature.

Another possible cause of protein deterioration in soybeans in storage is the occurrence of reactions between the proteins or the proteinogenous amino acids and the sugars, mainly pentoses, that occur in soybeans to the extent of about 10% (MacMasters, Woodruff and Klaas, '41). This sugar-amino acid reaction, the so-called "browning reaction," has been shown by Henry, Kon, Lea and White ('48) to account for the nutritive deterioration of the proteins of dried skim milk in storage at moisture levels of 5 to 7%, the point of attack being the  $\epsilon$ -amino group of lysine in the intact protein. Glycinin, the globulin of the soybean, has been shown to react in the same way on refluxing with glucose (Patton, Hill and Foreman, '48). The amino acids which seemed to be attacked by "browning" an intact protein are chiefly those containing functional nitrogen groups unattached in peptide linkages: epsilon amino (lysine), guanido (arginine), indole (tryptophan) and imidazole (histidine) groups. However, amino acids liberated from soybean proteins by the proteases present would be subject, presumably, to the same reaction. This is not an enzymic reaction, but it is one that is accelerated by heat; it would therefore occur both in the soybean sample stored whole and then autoclaved before being tested and in the sample autoclaved before storage. The smaller indicated protein impairment in the latter case may be a result of the "browning reaction" only.

#### SUMMARY AND CONCLUSIONS

The effect of storage at approximately 78°F. (25.5°C.) and at moisture contents of 6 to 12% on the nutritive value of the proteins of wheat (Turkey Red), corn (an open-pollinated and a hybrid variety) and soybeans (Illini) was determined. The storage periods lasted from 730 to 1,020 days. The corn and wheat samples were stored both as whole kernels and as meals ground to pass a 1 mm sieve. The soybeans were stored



as whole beans and as an autoclaved, defatted, and dehydrated meal. The beans stored whole were autoclaved, defatted, dehydrated and ground in the same manner before being tested.

The tests of the biological availability of the proteins ( $N \times 6.25$ ) of the stored seeds were carried out two or three times during the storage period by the nitrogen balance procedure developed in this laboratory, using growing albino rats as subjects. The utilization of the seed proteins in digestion was measured by the coefficient of true digestibility, making due allowances for the non-dietary nitrogen (metabolic nitrogen) in the feces. The utilization in metabolism was measured by the biological value, representing the percentage of the absorbed nitrogen retained for maintenance and for growth under standardized conditions of feeding.

The methods of testing used detected no consistent or significant deterioration in the digestibility or the biological value of corn and wheat proteins during storage for two to three years, either as whole kernels or as a ground meal.

Soybeans stored for 1,020 days as raw whole beans deteriorated definitely and significantly in both the digestibility and the biological value of their nitrogen (protein). Under the conditions of storage imposed, the average impairment in digestibility of nitrogen amounted to 10 percentage units, while the impairment in biological value averaged 13 percentage units. The total impairment in the nutritive value of nitrogen, as measured by the net utilization values, amounted to about 17 percentage units, representing a deterioration in the nutritive value of conventional protein ( $N \times 6.25$ ) of 28%. Since the preheating of soybeans prior to storage prevented this deterioration largely or entirely, the reactions responsible for observed changes are probably largely enzymic in character and involved in the respiration of the seed embryo, which makes up 92% of the whole seed. The possibility of sugar-amino acid involvement is discussed.

Working with samples stored for 1,020 days, methionine supplementation improved the biological value of both storage samples of soybeans, as it does that of raw or heated freshly

harvested beans; in the case of the samples stored raw and as whole beans, the average increase in biological value amounted to 6 percentage units, while for the preheated sample the increase averaged 20 percentage units. These facts permit the conclusion, first, that the damage inflicted by storage on the metabolic utilization of soybean proteins relates primarily to the cystine or methionine (or both) components of soybean proteins; and, second, that the greater storage damage in the unheated beans involves also other amino acids than cystine or methionine.

From the results of these and other studies it appears that the nutritive value of the proteins of cereal grains stored under conditions preventing insect infestation and mold growth (by lowering moisture content below a critical level) is not appreciably altered by seed respiration over long periods of time. This is not true, however, for the soybean, the proteins of which may suffer considerable nutritional damage after one year of storage. The distinction between the two types of seed may reside largely in the fact that the living part of the seed, the embryo, constitutes only a small part of the cereal seed but a predominant part of the legume seed.

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# A GROWTH FACTOR TRANSMITTED BY THE HEN THROUGH THE EGG TO HER PROGENY<sup>1</sup>

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That the cystine and methionine content of a hen's egg can be increased by changing the hen's diet from a ration in which corn is the chief source of protein to a high protein ration containing 20% casein or 20% soybean protein has been shown (Csonka et al., '47).

This study was undertaken to learn whether or not there would be any difference in the growth of chicks hatched from these eggs. Chicks from the eggs of hens fed high- and low-protein diets were reared together and fed identical rations. From our observations of these chicks, we conclude that the statistically significant differences in growth were not related directly to the increase in the cystine and methionine content of the eggs but were due solely to differences in their mothers' dietary proteins, the chief variable component in our study.

## EXPERIMENTAL PROCEDURE

We carried out two series of experiments, one in 1947 and the other in 1948. In each series, two pens of crossbred pullets in their first laying season were used. Each pen contained from

<sup>1</sup> This is the first article in a series dealing with the influence of dietary protein. A preliminary report was presented by the authors before The Federation of American Societies for Experimental Biology, 1949 annual meeting, held at Detroit, Michigan. Federation Proceedings, Part 1, Volume 8, page 193 (1949).

15 to 18 hens and one rooster. The chickens in each pen were fed rations differing in quantity and quality of protein.

The basal, low-protein diet (table 1) contained all the known nutrients in sufficient amounts for egg production and good hatchability, including approximately 9 gm of crude protein supplied mainly by yellow corn. The two high-protein diets were identical to the basal diet except that in one 20 parts of the corn meal were replaced by casein (commercial), and in the other 50 parts of the corn meal were replaced by soybean meal (commercial).

TABLE 1

*Composition of the basal diets fed to laying hens and to their chicks*

INGREDIENTS	HEN FEED (low-protein)	CHICK MASH
Yellow corn	87	50
Alfalfa leaf meal	5	12
Pulverized oats		30
Wheat meal		47
Soybean meal		47.5
Sardine meal		6
Limestone flour	2	2.5
B-Y feed (250 $\mu$ g riboflavin per gram)	0.5	0.92
Manganized salt (96% NaCl; 6% MnSO <sub>4</sub> )	0.5	1.0
Iodized salt	0.2	
D-sterol		0.075
Vitamins A and D feeding oil (2,000 I.U. of vitamin A and 400 A.O.A.C. units of D per gram)	0.3	
Steamed bone meal	4.5	

In each of the 9 experiments (table 2), hens in the two pens were fed simultaneously and their eggs collected for incubation. For instance, in experiment 1 a low-protein ration was fed to the hens in pen 1 and a high-protein ration containing casein to those in pen 2. In experiment 2 a similar feed was given to the same hens. In experiment 3 the rations were changed; hens that had been on the low-protein diet were put on a high-protein ration containing soybean meal, and those previously on a high-protein diet containing casein were fed a low-protein ration, plus dried cow manure.

This procedure for simultaneous feeding of the hens in the two pens and for changing the diets at specified intervals was continued in experiments 4, 5, 6, 7, 8, and 9 (1948). Whenever a hen's diet was changed, a period of three weeks was allowed to elapse before her eggs were used. This was done to eliminate the dietary effects of the previous ration on the eggs produced.

TABLE 2

*Various diets and the order in which they were fed to the hens whose eggs were collected for incubation*

SERIES	EXPERIMENT	PEN 1	PEN 2
		Ration fed to hen	Ration fed to hen
1 (1947)	1	Low-protein	High-protein-casein
	2	Low-protein	High-protein-casein
	3	High-protein-soybean	Low-protein, plus dried cow manure
2 (1948)	4	Low-protein	High-protein-casein
	5	Low-protein	High-protein-casein
	6	Low-protein	High-protein-casein
	7	Low-protein	High-protein-casein
	8	High-protein-soybean	Low-protein
	9	High-protein-soybean	Low-protein

Incidentally, the egg production of hens on the high-protein diets was higher than that of hens kept on low-protein feed. After a three-month period, some of the hens on low-protein rations stopped laying. This accounts for the small number of chicks from hens on this particular diet in the experiments in the 1948 series in which mash supplemented with dried cow manure or extract was fed.

The eggs laid by the hens in the two pens were gathered daily, marked for identification, and placed in a cooler at 55°F. Once each week the eggs were placed in forced-draft incubators that maintained a temperature of 100°F. and a relative humidity of 55%. At hatching time each chick was weighed and wing-banded.

Chicks hatched from eggs gathered in respective experiments were fed the chick mash (table 1) with or without sup-

plements. Chicks of hens fed low-protein rations served as controls in all instances. Chicks fed an identical ration, in any one experiment, whether in an experimental or control group, were caged together weekly (50 at a time) in the same compartment of an electrically heated battery brooder. Feed and water were supplied ad libitum. Individual chicks were weighed when 14 days and 28 days of age, but only the weights at 28 days were used for calculations.

The average gain of weight, shown in table 3, was calculated from the combined weights of all chicks of the same type reared in each experiment. In the statistical analysis (T value), comparison was made between the progenies of hens in pen 1 and pen 2.

Comparisons of experiments within the same pen in the same series furnished additional data.

#### RESULTS AND DISCUSSIONS

The series of experiments carried out in 1947 and again in 1948 showed that when the chicks were fed the same ration, consisting of mash only, those of hens fed high-protein rations containing casein gained more in weight than those of hens fed low-protein rations (table 3). In 1947 the average weight gains were, respectively, 240.8 gm and 196.9 gm, and in 1948 they were 203.5 gm and 179.0 gm. The differences were found to be highly significant. It should be emphasized again that all chicks in each experiment were caged together from hatching to their 28th day, and fed the same chick mash. The only difference observed between chicks with the two types of prenatal nutrition was that one gained weight faster than the other. The only difference in the food of their mothers was that the mothers in pen 1 received the low-protein diet in which the protein came mainly from corn, while those in pen 2 received the high-protein diet containing 20% casein.

The same conclusion was reached when the progeny of penmates in the 1948 series were compared. Chicks of hens fed high-protein rations containing casein gained an average of



TABLE 3

Average weight gain of chicks from eggs of hens fed various protein diets after feeding 25 days on mash alone and on mash with various supplements

VIAL OF SERIFS AND RATION FED TO MOTHER HEN	PLAN OF MOTHER HEN	RATION FED TO CHICKS									
		Mash only		Mash supplemented with							
				5% cow manure		Extract				0.2% liver fraction <sup>1</sup>	
		Number of chicks	Average weight gain	Number of chicks	Average weight gain	Number of chicks	Average weight gain	Number of chicks	Average weight gain	Number of chicks	Average weight gain
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)
<i>1937 series</i>											
Low protein	1	83	196.9	42	215.6						
High protein (casein)	2	109	240.8	39	191.6						
Difference			43.9		24.0						
T value			6.27 <sup>1</sup>		2.55 <sup>2</sup>						
<i>1938 series</i>											
Low protein	1	67	179.0	14	189.0	13	194.8	34	175.0		
High protein (casein)	2	81	203.5	40	162.0	10	196.5	98	171.0		
Difference			24.5		27.0		1.7		4.0		
T value			3.93 <sup>1</sup>		3.57 <sup>1</sup>		0.2 <sup>1</sup>		0.85		
<i>1937 series</i>											
Low protein, plus 5% dried cow manure <sup>2</sup>	2	33	180.3								
High protein (soybean) <sup>3</sup>	1	71	199.3								
Difference			19.0								
T value			1.88 <sup>1</sup>								
<i>1938 series</i>											
Low protein <sup>2</sup>	2	67	161.2	28	162.0						
High protein (soybean) <sup>3</sup>	1	122	151.0	65	164.0						
Difference			10.2		2.0						
T value			2.10 <sup>1</sup>		0.53						

<sup>1</sup> Statistically highly significant change in weight. Exceeds the 1% level according to Fisher's "t" table (Fisher, '36).<sup>2</sup> Statistically significant change in weight. Exceeds the 5% level according to Fisher's "t" table (Fisher, '36).<sup>3</sup> Ration was changed. Previously on high-protein (casein) ration.<sup>4</sup> Ration was changed. Previously on low protein ration.

203.5 gm, while those of hens fed low-protein rations gained only 164.2 gm. On the other hand, chicks of hens fed high-protein rations containing soybean meal showed less gain in weight than the control chicks. The differences are statistically significant. Results obtained with the progeny of pen-mates confirmed these findings. The chicks of hens fed high-protein rations containing soybean meal gained an average of only 154.0 gm and the chicks of hens fed low-protein rations 179.0 gm.

These results indicate that there was present in the diets fed a hitherto unrecognized growth factor. The carrier of this factor in the hens' diets would seem to have been casein as the most effective, corn as intermediate, and soybean meal as the least effective under our experimental conditions, since these were the only varying constituents in the rations.

Several tests were conducted in the course of these studies to establish a relationship between the growth factor found by us and the one which has been reported to be present in cow manure and in liver fraction "L." Bird et al. ('48) showed that the addition of 5% dried cow manure to their chick ration resulted in increased chick growth. However, in our studies, when 5% cow manure dried at 45°C. was added to the chick mash, chicks from hens fed the high-protein-casein ration grew more slowly and weighed less at 4 weeks than the controls. When comparison was made among the chicks of hens fed low-protein diets in the 1947 experiments, it was found that the chicks receiving mash supplemented with cow manure gained more weight than those receiving the mash only. In other words, the addition of dried cow manure to the chick ration: (1) caused a marked inhibition of growth in the high-protein-casein chicks; (2) had no effect on the growth of the high-protein-soybean chicks; and (3) caused a slight increase in the rate of growth of the low-protein chicks.

When chicks of hens fed low-protein rations containing dried cow manure were fed on mash only, their weight gains were not accelerated. This result indicated that the cow manure growth factor was not transmitted to progeny under the con-

ditions of this experiment. Under somewhat different experimental conditions, Rubin and Bird ('46) concluded that the factor was transmitted.

A sample of cow manure extract furnished by Dr. Bird (the preparation of which has been described by Rubin and Bird, '46) and added to the chick mash caused only a slight inhibition in the growth of chicks of hens fed high-casein rations. Seemingly, the inhibitory factor in this preparation had been mostly removed; the average gain of these chicks in 28 days amounted to 196.5 gm. In comparison, the chicks of hens fed high-casein rations, when given mash with 5% dried cow manure, gained only 162 gm; on mash alone the chicks gained 203.5 gm. The addition of cow manure extract to the mash produced accelerated weight gains in chicks of hens fed low-protein rations.

These results indicate the presence of a growth-inhibiting and a growth-promoting factor in dried cow manure, yet they do not explain why growth was inhibited in the chicks of hens fed high-protein rations containing casein but not in those of hens fed low-protein rations.

Wilson's liver fraction "L" is also supposed to contain a growth factor. However, when it was fed under our experimental arrangements no growth-stimulating effect was observed; the average gain in body weight both of chicks of hens fed low-protein rations and those of hens fed high-protein rations containing casein was practically the same. However, if we compare the average gain of high-protein-casein chicks in column 10, pen 2 (table 3), with that of high-protein casein chicks in column 4, same pen, the inhibitory effect of the liver fraction "L" and not the growth-promoting effect is found to be the prevailing one. Under our experimental conditions the inhibitory effect on the low-protein chicks was slight.

It is doubtful whether the agent causing accelerated growth in the high-protein-casein chicks is identical with those growth factors already reported in the literature in connection with casein feeding, such as factor X (Cary et al., '46), strepogenin (Woolley, '46), and factor S (Scott et al., '47). First, the

growth effect we describe was transmitted from the casein-fed hen through her egg to the chick; none of the chicks received casein in its diet. Secondly, we found no accelerated growth of chicks from hens fed a high-protein-soybean diet; on the contrary, with such a diet the rate of growth was actually less than the growth rate of the low-protein chicks (column 4). Strepogenin and factor S have been shown to be present in soybean meal (Woolley, '46; Scott et al., '47). Additional heating of commercially obtained soybean meal was necessary before the activity of factor X could be noted (Cary et al., '46). However, the results we obtained with liver fraction "L" definitely eliminate factor X as being responsible for the growth effect.

Our experimental arrangements were such as to exclude the possibility that any known deficiency was a factor in the results we obtained. The chicks whose growth served as a criterion were reared together and fed a diet adequate in all nutrient factors known to be essential for chick development. The explanation of the better growth of the high-protein-casein chicks, observed during the 28-day period, in comparison to the low-protein chicks must reside in the differences in the dietary protein fed to the mothers of the chicks. We want to make it clear that, in our opinion, the transmittal of the growth factor we observed is not a result of inheritance as such, but strictly one of nutrition. We believe that the growth factor is incorporated in the hen egg, which serves as a food supply for the developing embryo.

#### SUMMARY

A group of hens in two separate pens were fed, alternately, low-protein and high-protein rations. High-protein rations were obtained by adding soybean meal and casein, respectively, to the basal diet.

The eggs produced were incubated and the chicks (reared together) were fed a chick mash. Twenty-eight days after hatching their weight gains were recorded.

It was found that hens fed a ration containing 20% casein transmitted a growth factor through their eggs to the chicks.

With soybean proteins in the ration the same effect was not observed.

The addition of dried cow manure or Wilson's liver fraction "L" to the chick mash inhibited the growth of chicks from hens fed the high-protein-casein diet.

The addition of cow manure dried at 45°C. to the chick mash slightly accelerated the growth of chicks from hens fed a low-protein ration, but failed to affect the growth of chicks from hens fed a high-protein-soybean diet.

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# NUTRIENT ANALYSES OF UNITED STATES FOOD SUPPLIES

ANALYSES OF COMPOSITED SAMPLES OF OUR NATIONAL FOOD SUPPLIES  
AND OF THE WHITE FLOUR AND NON-WHITE FLOUR  
COMPONENTS THEREOF IN TERMS OF VARIOUS  
NUTRIENTS

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Advances in knowledge of the composition of available food supplies and potential food resources, in relation to man's food requirements in terms of specific nutrients, command ever increasing interest.

With very few exceptions, previous publications on assessments of specific nutrients provided in national food supplies have been based on computations derived by use of tables of "average or representative" composition of the individual food items involved. Obviously, both this method and the method of direct analysis possess certain advantages and disadvantages. Considerable interest, therefore, attaches to comparative results obtained by the two methods.

One of the more recent and most extensive assessments of United States food supplies includes computed estimates of the apparent per capita consumption of major foods in terms of their retail weights and computations of the specific nutrients carried therein (Bureau of Human Nutrition and Home Economics; '46). This report includes a table of computed val-

ues for 11 nutrients "available for consumption" per capita per day for the civilian population of this country for the years 1909-1945. Another recent report deals with computations of the *nutrient composition of national food supplies* (in terms of 11 nutrients) for the civilian populations, per capita per day, for the United States, Canada and the United Kingdom for the pre-war years 1943 and 1944 (Combined Food Board, '44). An attempt has also been directed toward a world food survey by the Food and Agriculture Organization of the United Nations ('46), which culminated in a report of computations of the caloric values and protein content of the pre-war food supplies of 70 countries.

Each of the three publications just cited refers to the disappearance of nutrients presumably supplied by the edible portions of the food involved. These computations include average deductions for inedible refuse but make no allowances for other ways that food or food-nutrients can be lost or wasted in homes and public eating establishments.

Seemingly the only published accounts of attempts to assess the various nutritive values of a national dietary by means of direct analyses, and with due allowances made for nutrient losses in cooking, are those of Williams and his co-workers. The two reports of this work (Lane, Johnson and Williams, '42; Cheldelin and Williams, '43) are concerned chiefly with thiamine and with the riboflavin, nicotinic acid and pantothenic acid contents, respectively, of the "average American diet" after all foods have been prepared for serving. On this basis, the average American diet was reported to carry about 0.8 mg of thiamine, 1.4 mg of riboflavin, 11 mg of nicotinic acid and 4.9 mg of pantothenic acid, per 2,500 cal., provided none of the white flour or bread component was enriched. Substantial increases in the thiamine, riboflavin, nicotinic acid and iron contents of the diet would perforce accrue by virtue of enrichment of the white flour component or of breadstuffs made with white flour. An estimate of the increments contributed in terms of the three vitamins in the enrichment ingredients based upon earlier enrichment standards (Federal Register,



'41) was computed by Williams and his associates, but each of these would require recomputation to be in accord with the upward revision of the standards for these nutrients in enriched flour (Federal Register, '43) promulgated subsequent to publication of the Williams et al. reports.

The present investigation was designed primarily to determine by direct analyses of composited sample food mixtures the various nutrient contents of our national dietary with and without such nutrient contributions as are potentially available in the form of enriched flour-converted products, all food items in the mixtures having been prepared for serving. Some further work was included to provide information on nutrient losses occasioned by conversion of enriched flour to enriched flour-converted products, and on those losses occasioned by the cooking of such other food items as are not generally eaten in the raw state.

Analyses of national food supplies, especially after the preparation of the food for serving, quite naturally provoke an inquiry as to the nutritional evaluation of the national dietary. Any resolution of such an inquiry presupposes the existence of some appropriate standard of reference for comparison, a matter which was not a subject of study in the observations reported here. However, the dietary allowances for 10 nutrients as recommended by the Food and Nutrition Board ('43, '45, '48) could, within the limits of a rough approximation, be computed in terms of daily per capita allowances by taking into account the general distribution of the population as regards age, sex and activity. These data, combined with knowledge of the nutritional contributions of national food supplies after all items therein have been prepared for serving, should at least provide an indication of whether a national dietary is potentially or inherently capable of meeting the recommended dietary allowances for such nutrients as were covered by the analyses. For the purposes of such an appraisal the authors of the present article have included weighted averages for the recommended dietary allowances in terms of specific nutrients for the estimated composition of the civilian population of the

United States as of July 1, 1944,<sup>1</sup> in accord with the latest revision of these allowances (Food and Nutrition Board, '48).

#### EXPERIMENTAL

##### *Proportions of various food items in a mixture considered representative of our national dietary*

In table 1 are listed the individual food items, together with the quantities of each, included in 3,000-cal. allotments of the complete food mixture analyzed as a representative sample of the edible portions of United States food supplies in recent years. In planning for the kinds and proportions of various food items to be used in the sample food mixtures, full use was made of the most recent annual publication of Agricultural Statistics (U. S. Department of Agriculture, '47), of a recent publication of the Bureau of Human Nutrition and Home Economics ('46), and of a paper on the average American diet by Lane, Johnson and Williams ('42). For the conversion of purchased weights of food into edible portions of food, considerable recourse was made to the tabulated data of Chatfield and Adams ('40).

All of the analytical data are given in terms of the quantities of the respective nutrients in 3,000-cal. allotments of the complete food mixture or of some designated component part thereof, whichever is specifically indicated. The 3,000-cal. base was selected by the authors because this allotment would appear to be very close to the apparent per capita consumption rate per day in this country for the combination of all foods as prepared for serving at the nation's tables.

Space does not permit our giving a detailed account of the basis for selecting the proportions of the approximately 75 different food items included in the complete food mixtures

<sup>1</sup> The composition of the civilian population of the United States corresponding to the various age-sex-activity classifications designated in the Food and Nutrition Board's listing of recommended dietary allowances was provided by Dr. Margaret Haywood, Bureau of Agricultural Economics, U. S. Department of Agriculture, through Dr. Frank L. Gunderson, during the latter's period of service as Secretary of the Food and Nutrition Board, National Research Council.

analyzed. In lieu of this, the reader's attention is called to the data in column 4 of table 1, where the authors have set forth the annual rates of apparent consumption of the foods included ("as purchased" basis), which would correspond to the 3,000-cal. allotment in terms of edible portions. A further judgment of the rationale of compositing the edible portions of the foods as indicated in table 1 can be made by comparison of the data in column 4 with data on apparent per capita consumption rates released in recent years by two Bureaus of the United States Department of Agriculture (see columns 5 and 6 of table 1).

One detail with regard to fats, other than fat-spreads, should perhaps be mentioned here, since in the present report the treatment of this item marks a distinct departure from that with regard to any other foodstuffs listed in table 1. Much of the cooking fat and fat rendered from fat cuts of meat, although purchased as food, does not customarily appear on American tables. This fact was well borne out in domestic fat-salvage programs during the World War II period and in surveys of food wastage made during 1917-19 as reported by Pearl ('20). For this reason, the authors have deleted from the food mixtures analyzed about 10 lb. of cooking fat per capita per year, as a conservative allowance for the amount of fat purchases which would not appear as food prepared for serving.

#### *Preparation of sample food mixtures*

The individual food items to be combined in the mixture representative of the national food supply were purchased in the local retail markets of Minneapolis in early July, 1948. The kinds and relative quantities of the various food items included in the complete food mixture are shown in table 1, columns 1 and 2 respectively. Multiples of the quantities of foods shown in column 2 were composited for analyses in terms of the constituents indicated in the following section on Methods of Analyses.

TABLE 1

*Kinds and relative proportions of individual food items included in mixtures representative of United States food supplies*

(Authors' data are based on 3,000-cal. allotments of the complete mixture and annual consumption rates corresponding thereto; comparative data are based on apparent annual per capita consumption rates)

FOOD ITEM	IN 3,000-CAL. ALLOTMENTS D.P. BASIS <sup>1</sup>	METHOD OF FOOD PREPARATION <sup>2</sup>	CORRESPONDING ANNUAL CONSUMPTION RATE, A.P. BASIS	ANNUAL APPARENT PER CAPITA (CIVILIAN) CONSUMPTION RANGE 1940-45 (Agricultural statistics) <sup>3</sup>	ANNUAL APPARENT PER CAPITA (CIVILIAN) CONSUMPTION RANGE 1940-45 (Estimated retail weights) <sup>4</sup>	
<i>Grain products</i>						
White flour, enriched	199.0	Made into bread and biscuits	100	}	147.4-164.2	
Whole wheat (100%) bread	7.8	.....	4 <sup>c</sup>			
Rye bread, commercial	5.2	.....	2 <sup>c</sup>			
Cornmeal, whole grain	14.3	Cooked in pressure saucepan	12			
Rice, white	6.5	Cooked in pressure saucepan	5			
Oatmeal, quick cooking	5.2	Cooked in pressure saucepan	4			
Prepared breakfast cereals	7.8	.....	6	5.7-6.3 <sup>b</sup>		
Total grain products	245.8		193	187.1-202.9 <sup>a</sup> (Excludes barley)	192-208	
<i>Dairy products (excl. butter)</i>						
Milk, whole fresh	360.0	.....	290	}	187.1-202.9 <sup>a</sup> (Excludes barley)	
Milk, evaporated	26.0	.....	46 <sup>7</sup>			
Milk, dry, defatted	10.2	.....	98 <sup>7</sup>			
Cheese, commercial	9.3	.....	75 <sup>7</sup>			
Total dairy products (excl. butter)	405.5		509 <sup>7</sup>	406.1-507.2 <sup>7,8</sup>	} 215-257 qt.	
Total fluid milk equivalent			237 qt.			
Eggs, total weight	38.1	Soft cooked	37	}	311-397 eggs	
Eggs, number			316 eggs			
<i>Flesh foods</i>						
Beef, fresh	15.6	Broiled	}	}	} 302-374 eggs	
Beef, fresh	35.2	Cooked in pressure saucepan				59 <sup>a</sup>
Pork, fresh	13.1	Broiled	}	}		62.7-80.1 <sup>a</sup>
Pork, fresh	14.3	Cooked in pressure saucepan				
Pork, cured (other than bacon)	13.1	Broiled	}	}		7.4-12.3 <sup>a</sup>
Lamb, fresh	5.4	Broiled				
Veal, fresh	5.3	Fried	8 <sup>b</sup>	..		..
Poultry, fresh	14.3	Cooked in pressure saucepan	19 <sup>a</sup>	..		..
Fish, fresh	18.5	Broiled	}	}		..
Fish, canned	5.4	None, juice discarded				
Total flesh foods, incl. fish	140.2		182 <sup>a</sup>	163.9-182.8 <sup>a,b</sup>	148-160	
Total flesh foods, excl. fish			155 <sup>b</sup>			
<i>Potatoes and sweet potatoes</i>						
Potatoes, white	136.8	Cooked in pressure saucepan, liquid discarded	191	}	125-133 19.3-21.6	
Sweet potatoes	17.2	Cooked in pressure saucepan	16			
Total white and sweet potatoes	154.0		147	146.9-154.6 <sup>a</sup>	137-146	
<i>Citrus fruits and tomatoes</i>						
Grapefruit	12.4	None	}	}	..	
Oranges	24.8	None				43

*Other vegetables and fruits*  
Beans, baked with pork, canned  
Beans, lima, canned  
Beans, green soup  
Beets  
Cabbage  
Cabbage  
Carrots  
Carrots  
Cauliflower  
Celery  
Corn, whole kernel, canned  
Lettuce, head  
Lettuce, canned  
Okra, canned  
Onions, bulb  
Peas, canned  
Peas, and beans, dried  
Pumpkin, canned  
Radishes  
Rutabagas  
Sauterkraut, canned  
Spinach  
Turnips

	6.2	Heated		
	6.2	Heated		
	12.6	Heated in pressure saucepan		
	12.6	Cooked in pressure saucepan		
	2.9	Liquid discarded		
	2.9	Cooked in pressure saucepan, liquid discarded		
	20.6	None		
	0.4	None		
	5.7	Cooked in pressure saucepan		
	1.8	None		
	5.3	Cooked in pressure saucepan, liquid discarded		
	2.5	None		
	16.0	Heated		
	13.6	None		
	4.4	Heated		
	26.3	Cooked in pressure saucepan		
	16.9	Heated		
	9.1	Cooked in pressure saucepan		
	9.1	Heated		
	2.5	None		
	3.9	Heated		
	6.6	Cooked in pressure saucepan		
	4.7	Cooked in pressure saucepan, liquid discarded		
	18.5	Cooked in open saucepan		
	30.0	None		
	2.6	None		
	21.2	None		
	6.0	None		
	4.8	None		
	6.0	None		
	14.3	None		
	6.5	None		
	5.2	Cooked in open saucepan		
	2.5	None		
	6.5	None		
	11.5	None		

Total other vegetables and fruits	314.4	322	330-395 <sup>a</sup>	315-370
<i>Fats</i>				
Fat (including fat spreads)	58.0	Part used in bread and biscuits	47	
Fat cuts, including bacon	9.2	Fried (fat used in cooking)	7	41.6-50.4 <sup>a</sup>
Total fat and fat cuts	67.2		54	60-71
Sugar, or sugar and syrup, total	104.3	Part used in cooking	108	89.4-116.5 <sup>a</sup>
<i>Meat products</i>				
Peanuts, roasted	1.9	None	2.0	
Chocolate	1.3	None	1.9	
Yeast, moist baker's	1.7			
Grand total	1586			

<sup>1</sup> Multiples of quantities shown in this column were used for composing food mixtures 1 and 2, and mixture 3 except for omission of the enriched flour component in the white bread and biscuits.  
<sup>2</sup> All cooking liquors, juices, and rendered fats included unless otherwise stated in this column.  
<sup>3</sup> Data in this column taken directly from or computed from figures released in "Agricultural Statistics" (U. S. Dept. of Agri., '47).  
<sup>4</sup> Data taken from "Nutritive value of the per capita food supply, 1909-45," Bureau of Human Nutrition and Home Economics (46).  
<sup>5</sup> This figure refers to average weight of flour in the type of bread indicated.  
<sup>6</sup> Range of tons during 5-year period.  
<sup>7</sup> Weight of fluid whole milk equivalent to product indicated.  
<sup>8</sup> Computed by subtraction of fluid milk equivalent of butter from total fluid milk equivalent of dairy products.  
<sup>9</sup> Dressed weight basis.

All non-perishable food items included in the food mixtures were assembled one to two days preceding laboratory preparation of the mixtures. All perishable items were delivered to the laboratory in the late afternoon of the day immediately preceding preparation of the mixtures, and were stored overnight under refrigeration ( $4.5^{\circ}\text{C}$ ). Cooking, according to the methods indicated in column 3 of table 1, and other customary preparations of the various purchased food items, were begun early the following day according to a carefully prearranged schedule of timing, so that all items would be ready for mixing and blending at approximately the same time. A large share of the vegetable cookery was done in pressure saucepans, which further facilitated completing the preparation of these foods on schedule. As is indicated in table 1, expressed juices as well as cooking liquids were included in the sample mixtures except for cooking liquors associated with a few strongly flavored vegetables.

The blending of the individual food samples was accomplished by subjecting the specified kinds and quantities of food items to trituration in the glass receptacle of a motor-driven food blender. Except for such analyses as were initiated immediately after conclusion of the blending operation, analyses were made on aliquots of the mixtures which had been quick-frozen immediately after the blending of the food mixtures, and stored at a temperature of  $-29^{\circ}\text{C}$ .

The following food mixtures were assembled for analysis from single lots of purchased food items:

Mixture 1. Complete food mixture composed of the individual food items of the kinds and in the quantities specified in table 1 and with cooking procedures confined to the enriched flour component, 50% of this flour having been made into yeast-raised bread and 50% into baking powder biscuits.

Mixture 2. Complete food mixture, the same as mixture 1 except that all food items were prepared for serving prior to the blending of the individual food items.

Mixture 3. Identical to mixture 2 except that the flour, leavening agents, salt, yeast food and malted grain in all the enriched flour-converted products were excluded.

In addition to analyses of the mixtures described above, separate analyses were made on an aliquot of the enriched flour sample which was purchased along with other items at a local market and employed in making the bread and biscuits included in mixtures 1 and 2.

Since one of the objectives of this study was to determine the *potential* contributions of enriched flour to the national food supply, rather than those of some arbitrarily selected or assumed proportion of enriched and unenriched flour, the entire white flour-converted components in mixtures 1 and 2 were made with enriched flour. The selection of bread <sup>2</sup> and biscuits <sup>3</sup> as carriers of the nutritive contributions of the enriched flour component in mixtures 1 and 2 is, of course, a decided simplification of the many uses to which white flour is put in our national dietary. However, the authors considered that this simplification in procedure would yield about average nutritive values for such heat-labile nutrients as would be involved in the wide assortment of uses to which white flour is currently put in this country.

The total quantities of cooking fats and of sugar (sucrose) incorporated in *each* of the three food mixtures were the same whether portions of these two items did, or did not, appear as ingredients of bread and biscuits. The remainder of the non-flour ingredients used in making the bread and biscuits (yeast, malted grain, yeast-food, salt and the phosphate type of baking powder) were included only in mixtures 1 and 2, wherein the flour components were represented as enriched flour-converted products. This procedure was adopted after due consideration of the distortion of analytical results to be anticipated by introducing so questionable a procedure as

<sup>2</sup> Bread formula (all amounts in grams): Enriched flour 100, water 64, hydrogenated fat 3.5, sugar 5, salt 2, yeast 2, malted grain 1, yeast food 0.33.

<sup>3</sup> Biscuit formula (all amounts in grams): Enriched flour 100, hydrogenated fat 15.38, baking powder 14.8, sugar 2.50, salt 2, water 85.

having live yeast and unspent baking powder intimately associated with the constituents of mixture 1. Neither of these substances is normally associated in human dietaries with other than flour-conversion products.

All cooking procedures applied to either vegetables or meats were conducted with the objective of retaining maximum nutritive values. The reader will recognize this as a departure of indeterminate magnitude from average cooking practices in kitchens throughout the nation. It was decided that any arbitrarily selected variations with regard to conservation of nutrients would have introduced a situation very difficult, if not impossible, to evaluate, and would have been entirely inconsistent with the over-all objective of investigating the potential maximum of nutritive values in national food supplies.

The beef, pork and lamb samples included in the mixtures were selected in each case as representing, in our judgment, medium fatness and good quality. In so far as it was possible to do so, individual fruit and vegetable items were included in the food mixtures in the form or forms (fresh, canned, dried) most widely purchased by consumers. A few of the less common fruits and vegetables, grown and consumed in this country but not included because of their inaccessibility, were replaced by items having similar usages. This statement is made in explanation of the fact that the food items incorporated in the complete food mixtures (table 1) show omissions of some of the less common or regionally restricted fruits and vegetables. It was obviously impossible to select absolutely *identical* samples from retail purchase lots of such structurally inhomogeneous items as meats and vegetables for inclusion in the raw and prepared food mixtures, respectively. However, maximum care was taken to secure as nearly matched samples as visual perception permitted.

### *Methods of analysis*

The methods of analysis used in determining the quantities of various constituents in food mixtures 1, 2 and 3 and in the enriched flour are indicated in table 2.



The analyses for thiamine, riboflavin and nicotinic acid were initiated immediately following the blending of the food mixtures. The remainder of the analyses were made on aliquots of the same food mixtures which had been quickly frozen and stored in the frozen state at a temperature of  $-29^{\circ}\text{C}$ .

TABLE 2

*Analytical methods employed in the determination of various constituents in enriched flour and in food mixtures 1, 2 and 3*

CONSTITUENT	METHOD
Nitrogen	Kjeldahl method as outlined by Assn. Offic. Agri. Chem. ('45), p. 26.
Protein quality	Nitrogen balance (using young rats 50-60 gm in weight) in terms of whole egg protein replacement value; Sumner, Pierce and Murlin ('38).
Ash	Assn. Offic. Agri. Chem. ('45), p. 238.
Crude fiber	Assn. Offic. Agri. Chem. ('45), p. 409.
Fat	Acid hydrolysis as outlined by Assn. Offic. Agri. Chem. ('45), p. 240.
Calcium	Assn. Offic. Agri. Chem. ('45), p. 415.
Phosphorus	Ammonium vanado-molybdate method as adapted to cereal products by the General Mills analytical research staff, based on methods of Murray and Ashley ('38) and Willard and Center ('41).
Iron	$\alpha$ , $\alpha$ -dipyridyl method, Am. Assn. Cer. Chem. ('47), p. 47.
Potassium	Volumetric chloroplatinate method of Gerritz ('42); also flame photometer method conducted for us by the Laboratory of Vitamin Technology, Chicago, Ill.
Thiamine	Am. Assn. Cer. Chem. ('47), p. 114.
Riboflavin	Am. Assn. Cer. Chem. ('47), p. 121.
Nicotinic acid	Microbiological method of Andrews, Boyd and Gortner ('42).
Pyridoxine	Yeast growth method of Atkin et al. ('43), conducted for us by the Laboratory of Vitamin Technology, Chicago, Ill.
Pantothenic acid	Microbiological method of Skeggs and Wright ('44), conducted for us by the Laboratory of Vitamin Technology, Chicago, Ill.; same method modified to include clarase digestion, conducted for us by Food Research Laboratories, Inc., Long Island City, N. Y.
Moisture	Assn. Offic. Agri. Chem. ('45), p. 382, modified to include overnight drying.
Vitamin E	Biological assay following the principle of Palmer's ('37) method.

In the cases of moisture, ash, nitrogen, fat, crude fiber, calcium, phosphorus, iron and potassium, two or more entirely separate analyses were made for each constituent, with each analysis including determinations made in either duplicate or triplicate. The values shown in this report represent simple averages of the two or more sets of analyses made at different times.

The thiamine analyses were made in triplicate under the supervision of two analytical chemists working independently of one another. The values for thiamine represent simple averages of these three separate analyses.

The analyses for pyridoxine and pantothenic acid and for potassium by the flame photometer method were, as is indicated in table 2, conducted for us in outside laboratories.

Appreciable amounts of water were required to be added to the food mixtures during the blending process to facilitate mixing. Moisture determinations made on these diluted mixtures, therefore, served to provide the means for computing the contributions of the various constituents to the total weights of the mixtures, but no attempt was made to make direct determinations of the moisture contents of the undiluted food mixtures themselves.

## RESULTS

In table 3 are given the results of the analysis of samples of the three composited food mixtures and the enriched flour described earlier. The data shown in columns 2 to 5 inclusive refer to the quantities of respective constituents found in approximately 3,000-cal. allotments of the complete food mixtures (or indicated component parts thereof) representative of our national food supplies in recent years.

A 3,000-cal. allotment of United States food supplies corresponds roughly to about 1.6 kg of fresh foods (E. P. basis) and to approximately 0.7 kg of edible dry food-substance. Thus, in spite of the lack of direct determination of the moisture contents of the undiluted food mixtures, it can nevertheless be stated that our over-all national food supplies are comprised,

very roughly, of 40% and 60% of dry food substance and moisture, respectively.

TABLE 3

*Analytical data showing nutrient composition of food mixtures 1, 2 and 3 (see text) and of uncooked enriched flour ingredient included in mixtures 1 and 2 in the form of bread and biscuits. (Computed data in column 6 included for comparative purposes)*

CONSTITUENT ANALYZED	COMPLETE NATIONAL DIETARY WITH ALL ITEMS EXCEPT ENRICHED FLOUR UNCOOKED (Mixture 1)	MIXTURES PREPARED FOR SERVING		ENRICHED FLOUR COMPONENT (199 gm)	COMPUTED RECOMMENDED DIETARY ALLOWANCES PER CAPITA PER DAY <sup>1</sup> (1948 revision basis)
		Complete national dietary (Mixture 2)	Mixture 2 with enriched flour ingredient omitted (Mixture 3)		
Protein					
(N × 6.25), gm	91.	93.	67.		64.
Protein					
(N × 5.7), gm				21.	..
Protein quality					
(% egg protein replacement)	81.	84.	88.	65.	..
Ash, gm	23.1	23.9	11.8	0.8	.
Fiber, gm	8.	9.	7.	0.4	..
Fat (acid hydrolysis), gm	94.	99.	92.	2.4	
Carbohydrate (by difference), gm	447.	447.	280.	151.	
Calories	3000.	3050.	2215.	708.	2610.
Calcium, gm	1.03	1.04	0.78	0.03	1.1
Phosphorus, gm	2.95	3.06	1.12	0.18	
Iron, mg	17.7	20.4	9.8	7.5	11.7
Potassium (chem. method), gm	3.0	3.0	2.7	0.2	
Potassium (flame photom.), gm	3.2	3.0	2.9	0.1	
Thiamine, mg	2.03	1.78	1.09	0.96	1.2
Riboflavin, mg	2.13	2.14	1.56	0.48	1.6
Nicotinic acid, mg	20.7	20.2	13.2	8.2	12.6
Pyridoxine, mg	1.7	1.5	1.4	0.1	
Pantothenic acid, mg	8.1	8.0	5.1	1.1	
Dry weight of food mixture, gm	663.	672.	458.	175.	.

<sup>1</sup> For source and derivation of these figures, see text, pages 497 and 498.

According to the analytical data set forth in table 3, the civilian population of the United States derive about 12% of the total caloric value of their food from protein, about 28% from fat and about 60% from carbohydrates.

The protein in composited samples of our over-all national food supply, all items in the mixture having been prepared for serving, was observed to have a whole egg protein replacement value of 84%, while the protein in the same diet minus the entire white flour component showed a whole egg protein replacement value of 88%. The proteins of the white flour, the white bread and the biscuits showed no significant differences in biological quality, each exhibiting a whole egg protein replacement value of approximately 65%. Thus white flour proteins, providing about 25% of the total protein in our national dietary, alter the over-all protein quality of the dietary in only small degree from that found in the composited non-flour component.

The calcium and phosphorus contents of food mixture 1, as well as of food mixture 2, were approximately 1.0 gm and 3.0 gm, respectively. The calcium and phosphorus contents of our national dietary, exclusive of baking powders, yeast foods and all leavening agents carried by phosphated and self-rising flours, would be of the order of 0.80 gm and 1.3 gm, respectively, per 3,000 cal. It is clear, however, that various materials other than yeast which are commonly used as leavening agents contribute significantly to the available dietary supplies of calcium and phosphorus of the American people. These also include phosphated and self-rising types of white flour, widely consumed in southern and southwestern regions of the United States, neither of which was included in the samples of the national dietary analyzed by us because of the apparent lack of reliable records of consumption rates on a nationwide scale.

The iron content of United States food supplies amounted to about 18 to 20 mg per a 3,000-cal. allotment of food, which quantities include iron derived from tap water used in cooking and from contamination by cooking utensils.

Except in the case of thiamine our observations have not indicated any *necessity* for the incurrence of B vitamin losses of much over-all practical significance as a result of simple cooking procedures. The thiamine value of a 3,000-cal. allotment of United States food supplies prior to preparation of the individual food items for serving was observed to be about 2.3 mg (2.03-[1.78-1.09] + 0.96); the same quantity of food after preparation for serving was observed to carry about 1.8 mg of thiamine, or about 20 to 25% less than was present in the total raw foods. These data refer to the complete food mixtures when the entire white flour component is enriched and when all cooking procedures are conducted with strict attention to conservation of the maximum thiamine values. The unavoidable losses in thiamine occasioned by preparation of the foods for serving were sustained in approximately equal proportions by the enriched flour and non-flour components.

The white flour component, assuming all of it to be enriched, would provide (in addition to about one-fourth of the total protein, around one-third of the carbohydrate and from 2 to 3% of the fat) approximately the following percentages of the other nutrients in United States food supplies after careful preparation of the foods for serving: 40 of thiamine, 25 of riboflavin, 35 of nicotinic acid and at least 45 of iron. The white flour component also provides appreciable amounts of calcium, phosphorus, potassium, pyridoxine and pantothenic acid, although the practical importance of these would appear to be minor except in dietaries where flour is consumed in more-than-average quantities.

The results of attempts to assess the vitamin E values of the mixtures representing our national food supply before and after preparation were not entirely satisfactory because: (a) the quantities of the food mixtures required to be fed over the 10-day supplementary feeding period greatly lowered, and at some levels of feeding entirely displaced, consumption of the vitamin E-free ration; and (b) it was quite obvious that major destruction of the vitamin E activity in food mixture 1 had taken place within the 15-day interval between preparation

of the mixtures and completion of the 10-day feeding period. The results, in comparison with those found with rats consuming the vitamin E-free ration supplemented with different levels of alpha-tocopherol, indicated a potency equivalent to about 18 mg of alpha-tocopherol per a 3,000-cal. allotment of our national food supplies after preparation for serving, and only about one-third of this amount in the same quantity of food mixture 1. Obviously the vitamin E determination made on food mixture 1 is not valid. To what extent the destruction of vitamin E or the displacement of the vitamin E-free ration during the 10-day feeding period may have affected the results obtained for mixture 2 cannot be assessed, but it is obvious that major destruction of the vitamin E activity had taken place in food mixture 1 despite low temperature storage ( $-29^{\circ}\text{C}.$ ) of the material prior to feeding. In view of the obvious rapid destruction of vitamin E activity in at least one of the food mixtures, this work was not followed up with attempts to reassess the vitamin E activity, using fat-solvent extracts of the mixtures.

Difficulties analogous to, or even more serious than, those encountered with vitamin E could reasonably be expected in attempts to determine vitamin A and ascorbic acid values in composited and blended food mixtures, and for this reason these latter two vitamins were left entirely out of consideration in this study.

#### DISCUSSION

Since all of the analytical data presented in table 3 for enriched flour and for composited food mixtures 1, 2 and 3 represent observations for which samplings of the same lots of each food item concerned were employed, these data would seem to invite, or at least lend themselves quite logically to, various types of computable interrelationships. In this connection, however, the authors wish to emphasize that the data shown for enriched flour and for the three food mixtures are primary sets of data derived in each case by separate analyses. The well-known concept of additive experimental error, therefore,

cannot be disregarded as a factor of potential importance in mathematical manipulations of these data.

On the basis of data presented here and elsewhere there should be no difficulty in making comparisons of the nutrient compositions of United States food supplies (prepared for serving) with and without benefit of enrichment of the white flour component. Obviously the substitution of unenriched flour for an equal weight of enriched flour would not alter the nutrient composition of our national dietary except as regards nutrients included as enrichment ingredients. Lane et al. ('42) and Cheldelin and Williams ('43) have already reported in earlier issues of this Journal the thiamine, riboflavin and nicotinic acid values of United States food supplies when the entire white flour component was unenriched. The data of these authors show a 3,000-cal. allotment of our national food supplies to contain 0.92 mg thiamine, 1.7 mg riboflavin and 13 mg nicotinic acid; which findings in the light of various types of information assembled in this laboratory, appear to be thoroughly representative, yet stand in marked contrast to the respective values for these three nutrients in our mixture 3 (see table 3), wherein the entire white flour component was enriched.

It will be noted from data presented in table 3 that, even after making due allowance for the difference in protein factors used in expressing the protein content of the enriched flour and non-flour components, the sum of the protein in these two fractions is slightly less than the protein content of either of the complete food mixtures. The shortage is presumably to be accounted for by sampling errors and by the relatively small protein contributions of yeast and malted grain, which were included only in complete food mixtures 1 and 2.

Any rational evaluation of the data presented in this report with the objective of arriving at an opinion or judgment of the nutritive adequacy of our over-all national food supplies must take into account the fact that our data make no allowances either for food wastage in homes and public eating establishments, other than for 10 lb. rendered or cooking fat

per capita per year, or for unnecessary losses in food nutrients occasioned by over-cooking or by holding foods at elevated temperatures after cooking. The only report of a large-scale investigation of food wastage known to the authors is that of Pearl ('20), who estimated that during the years 1917-19 at least 25% of the fat, probably as much as 20% of the carbohydrate, and about 5% of the protein in our national food supplies appeared in the nation's garbage.

In connection with Pearl's observations it might also be of interest to point out that there appears to be a decided trend toward over-consumption or wastage of food as the family income and the amount of income expended for food rise above family subsistence levels (Booher, '48). Moreover, according to the reports of several investigators (Briant et al., '46a, b; Goodhart, '43; Heller et al., '43; Nagel and Harris, '43; Sarett et al., '46; Streightoff et al., '46; Wertz and Weir, '44), the degree of retention of specific heat-labile nutrients in foods prepared and served in public eating places bespeaks an urgent need for improvement in cooking procedures in order to avoid unnecessary losses of thiamine, ascorbic acid, riboflavin and nicotinic acid values in foods. Possession of adequate food supplies alone will, obviously, not assure an adequate national dietary.

By combining the data shown in table 3 for enriched flour, mixture 1 and mixture 3, one can arrive at figures for the respective nutrients present in the composited food supplies in the raw or unprepared state. According to our observations, the *minimum* loss of thiamine to be anticipated as a result of careful preparation of our national food supplies is on the order of 20 to 25% of that in the original raw food, while losses of riboflavin, nicotinic acid, pyridoxine and pantothenic acid under the same conditions are of no practical significance. These observations, however, do not in the least conflict with the observations of others that maximum conservation of heat-labile nutrients falls far short of being common practice in our nation's kitchens.



As the result of data presented here, as well as the findings in various other publications herein referred to, it would seem appropriate to conclude that our over-all national dietary is inherently and *potentially* capable of providing the recommended dietary allowances (recommended intakes) of each of the 7 specific nutrients applicable to studies reported here. This potentiality, however, is contingent upon negligible food wastage, maximum conservation of food nutrients in the processes of food preparation, and use of a large proportion of the white flour component in the form of enriched flour (or an equivalent thereof).

#### SUMMARY

Analyses of food mixtures representative of United States food supplies in recent years in terms of proximate composition, protein quality, calcium, phosphorus, iron, potassium, thiamine, riboflavin, nicotinic acid, pyridoxine, pantothenic acid and vitamin E are herein reported.

The data presented provide the means for assessing the levels of these nutrients in the white flour and non-white flour components of our national dietary prior and subsequent to the application of simple cooking procedures to the individual foods included, the white flour component being represented, in accordance with the stated objectives of this investigation, as enriched flour.

It is demonstrated that the relatively large losses of thiamine and the lesser losses of other B vitamins reported by earlier investigators to have been encountered in various public or communal eating establishments represent, in large degree, unnecessary losses of food value.

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# THE UTILIZATION OF CAROTENE FROM DIFFERENT SOURCES BY LAYING CHICKENS<sup>1</sup>

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FOUR FIGURES

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A number of investigators have shown that animals vary in their ability to convert carotenoid pigments into vitamin A. Differences have also been reported in the utilization by animals of carotenoids obtained from different sources. The above variations give rise to differences in the biological activity of carotenoids as regards their vitamin A potency. Callison and Orent-Keiles ('47) have shown that carotene derived from carrots is not utilized in the human body to the same extent as carotene obtained from other sources. Guggenheim ('44), working with rats, found that only from 33% to 67% of the carotene of plant origin is utilized as compared to the carotene in beef liver; the carotene from lettuce was found to be an exception, being utilized equally as well as that derived from liver. Hickman et al. ('44) and Harris et al. ('44) showed that natural mixed tocopherols enhanced the growth-promoting power of vitamin A and markedly influenced the vitamin A activity of carotene in the rat.

In the present investigation a study was made to compare the utilization by the chicken of vitamin A-active substances in the form of  $\beta$ -carotene, vitamin A esters from fish liver oil,

<sup>1</sup> Colorado Agricultural Experiment Station, Scientific Series Article no. 296.

and the naturally occurring carotenoids in fresh alfalfa and dehydrated alfalfa meal. The criteria for carotene utilization are based on liver storage, blood levels, and egg content of vitamin A and carotene. Frey et al. ('47) have presented data indicating that serum levels of vitamin A in cattle are a good index of vitamin A status in this animal. Within a wide range, liver storage was found to be approximately proportional to intake. The above observations have been confirmed by Hartzler ('48), using human subjects. Hoefer and Gallup ('47) compared the levels of vitamin A in the blood and livers of fattening lambs, the sources of carotene being alfalfa meal and carotene concentrates. Alfalfa meal was found to maintain a higher level of vitamin A in the blood than did carotene concentrates. Ross et al. ('48) found that Holstein heifers were capable of utilizing vitamin A alcohol and the natural esters of vitamin A more efficiently than carotene concentrates.

#### EXPERIMENTAL DESIGN

One hundred white Leghorn hens, approximately 9 months of age, were selected from a single flock on the basis of uniformity of size and condition. The birds were divided equally in 5 pens of the same size and shape. From February 4, 1948, to March 7, 1948 (33 days), they were fed the following basal diet, low in vitamin A activity: ground barley 37%, pulverized oats 25%, wheat gray shorts 20%, soybean oil meal 5%, meat and bone scrap 5%, pulverized limestone 3.5%, dried yeast 3%, steamed bone meal 1%, iodized salt 0.5%, D-activated animal sterol (2,000 AOAC U/gm) 25 gm %, and manganese sulfate 5 gm %. Blood samples were taken by wing puncture from 15 birds for vitamin A and carotene analysis at the time they were placed on the basal ration. On March 4 (30 days), two birds from each pen were sacrificed to determine liver stores and blood levels of vitamin A and carotene. Body reserves of the two constituents were found to have been sufficiently depleted to permit placing the remaining birds on the various supplements containing 2,000 I.U. of vitamin A per pound of ration consumed. The rations receiving their vitamin

A activity from carotene contained 1.20 mg of carotene per pound (assuming 0.60  $\mu$ g of carotene to be equivalent to 1.0 I.U. of vitamin A). The rations receiving their vitamin A activity from fish liver oil contained vitamin A esters equivalent to 0.60 mg of vitamin A acetate per pound, as measured from a standard curve assuming 1.0  $\mu$ g of vitamin A acetate to be equivalent to 3.33 I.U. of vitamin A. The following vitamin A active rations were fed: pen 1, basal diet; pen 2, basal diet +  $\beta$ -carotene in cottonseed oil; pen 3, basal diet + vitamin A esters from fish liver oil; pen 4, basal diet + fresh alfalfa; and pen 5, basal diet + dehydrated alfalfa meal. The experimental feeding period was terminated May 18, 1948 (72 days).

The supplements containing the vitamin A-active substances were fed Monday, Wednesday, and Friday of each week at 11 A.M. The calculated amount of vitamin A-active substance was weighed and mixed with a small amount of the basal ration and water to form a crumbly wet mash. The mash was placed in troughs and consumed within an hour after feeding. The fresh alfalfa used was cut in September, 1947, packed in cardboard containers, sealed, and quick frozen. When ready to be used the frozen alfalfa was ground in a food chopper, packed in tin cans, and again quick frozen. All supplements containing vitamin A activity were analyzed at frequent intervals for vitamin A and carotene content.

Blood samples were obtained by heart puncture from three birds in each pen on April 8. On April 26 one-half the birds from each pen were sacrificed to determine liver stores and blood levels of vitamin A and carotene. The remaining birds were sacrificed May 18. The whole liver was removed from each bird at the time it was killed, wrapped in wax paper, and quick frozen. At the beginning of the experiment three birds from each pen were selected and the yolk of each egg laid analyzed for vitamin A and carotene content. At the beginning of the experimental feeding period it was necessary to select several new birds to replace those which had ceased producing eggs. Trap nests were used for all birds and a record was

kept on egg production and ration consumption during the experimental period of 105 days.

The methods of analysis for vitamin A and carotene in blood and liver tissue were those described by Frey and Jensen ('46). The carotene content of egg yolk and alfalfa was determined by the "phasic" method given in an Association of Official Agricultural Chemists publication ('45). The vitamin A content of egg yolk was determined as in liver, using the petroleum ether fraction following separation of the carotene. Chromatographic methods for the separation of carotene in egg yolks have not proved satisfactory. For this reason it was thought advisable to use the "phasic" method for all carotene determinations other than liver and blood. Values for the carotene content of alfalfa, using chromatographic methods of analysis, compared favorably with the values found by the "phasic" method.

#### RESULTS AND DISCUSSION

The purpose of the preliminary feeding period of 33 days on the basal diet was to deplete the birds of their vitamin A and carotene reserves. The blood level at the beginning of the preliminary vitamin A depletion feeding period was  $99.8 \pm 59.3$   $\mu\text{g}$  of vitamin A and  $211 \pm 183$   $\mu\text{g}$  of carotene per 100 ml of serum. At the beginning of the experimental feeding period the blood level had dropped to  $28.5 \pm 9.7$   $\mu\text{g}$  of vitamin A and  $33.1 \pm 19.9$   $\mu\text{g}$  of carotene per 100 ml of serum. The latter values indicate fairly depleted reserves of the two blood constituents.

Figures 1 and 2 show the blood level and liver values of vitamin A and carotene during the experimental feeding period of 72 days. Complex variance analysis studies were made of vitamin A and carotene values for blood and liver samples obtained on April 26 and May 18. No significant difference between these dates was found within pens. However, highly significant differences between pens were found between these dates. Following are the the minimum differences required for significance (M. S. D.) between pens, for blood levels and liver



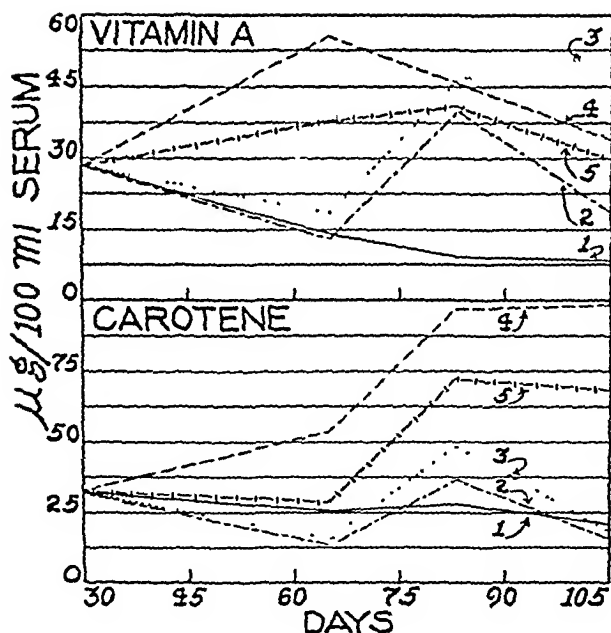


Fig. 1 Blood levels of vitamin A and carotene. 1. Basal ration. 2. Carotene supplement. 3. Vitamin A supplement. 4. Fresh alfalfa supplement. 5. Dehydrated alfalfa meal supplement.

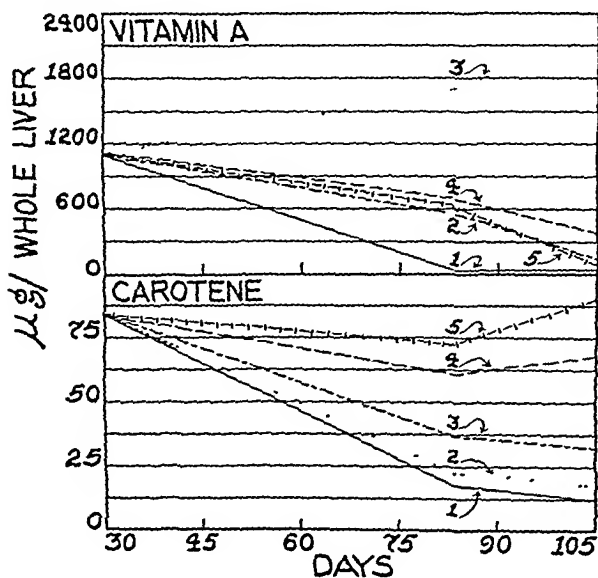


Fig. 2 Liver stores of vitamin A and carotene. 1. Basal ration. 2. Carotene supplement. 2. Vitamin A supplement. 4. Fresh alfalfa supplement. 5. Dehydrated alfalfa meal supplement.

reserves of vitamin A and carotene for April 26 and May 18: At the 0.01 level, 21.1 for blood vitamin A, 41.0 for blood carotene, 30.9 for liver vitamin A, and 31.5 for liver carotene; at the 0.05 level, 15.9 for blood vitamin A, 30.8 for blood carotene, 23.3 for liver vitamin A, and 23.7 for liver carotene. Table 1 gives the levels of significance, obtained from "t" values, for blood and liver reserves of vitamin A and carotene between pens, for May 18.

TABLE 1

*Levels of significance, obtained from "t" values, for blood and liver reserves of vitamin A and carotene, between pens, after 72 days on the experimental diet (All birds were fed a preliminary vitamin A-depletion ration before being placed on the experimental diet)*

PENS <sup>1</sup>	BLOOD		LIVER	
	Vitamin A	Carotene	Vitamin A	Carotene
1-2	0.001	0.40	0.10	0.01
1-3	0.01	0.90	0.01	0.05
1-4	0.001	0.01	0.01	0.001
1-5	0.001	0.10	0.05	0.001
2-3	0.02	0.80	0.01	0.001
2-4	0.02	0.05	0.10	0.001
2-5	0.02	0.10	0.50	0.02
3-4	0.20	0.05	0.02	0.001
3-5	0.10	0.10	0.01	0.01
4-5	0.50	0.50	0.05	0.30

<sup>1</sup> Pen 1: Basal diet only.

Pen 2: Basal diet +  $\beta$ -carotene in cottonseed oil.

Pen 3: Basal diet + vitamin A esters from fish liver oil.

Pen 4: Basal diet + fresh alfalfa.

Pen 5: Basal diet + dehydrated alfalfa meal.

The blood levels of carotene and vitamin A in the negative control pen, 1, did not decline significantly from March 4 to April 8, representing, respectively, 30 and 65 days on the basal ration. This indicates that minimum blood levels had been attained, which would not change until liver stores were further depleted. By April 26 (93 days) the liver reserves of vitamin A and carotene of the birds in pen 1 were practically

gone and blood levels were not significant, since the values were within the limits of error of the analytical methods used.

The birds in pen 3, receiving vitamin A, showed the same carotene values in the blood and liver as the birds in pen 1 receiving the basal ration. However, they showed the highest blood and liver vitamin A values, the liver values indicating that storage had occurred even though the blood level was only about one-half the value at the beginning of the experiment.

The birds in pen 2, receiving carotene, showed approximately the same blood carotene content as the control group in pen 1, even though liver reserves were somewhat higher. The blood and liver vitamin A values in pen 2 were significantly higher than in pen 1 after 53 days of the experimental feeding period, but were not significantly higher after 72 days. On the other hand, both fresh alfalfa and dehydrated alfalfa meal were more effective than  $\beta$ -carotene in maintaining body reserves of carotene. The carotene content of the blood and liver of the birds receiving alfalfa was higher than that of any of the birds in the other three pens at both 53 and 72 days. At 72 days the blood levels of vitamin A for the birds receiving alfalfa were higher than for the birds receiving carotene but lower than for the birds receiving vitamin A. The liver reserves of vitamin A of the birds receiving alfalfa closely approximated the values for the birds receiving carotene. The liver reserves of vitamin A for the birds receiving fresh alfalfa were significantly higher than those for the birds receiving dehydrated alfalfa meal, and significantly lower than those for the birds receiving vitamin A. None of the carotene-containing supplements approached vitamin A in effectiveness in restoring vitamin A reserves in the liver. It thus appears that  $\beta$ -carotene in cottonseed oil or the vitamin A-active substances in fresh alfalfa or dehydrated alfalfa meal are less effectively utilized as vitamin A than is vitamin A obtained from fish liver oil.

The tocopherols have been shown to be effective in aiding the utilization of carotene in the animal body (Hickman et al.,

'44; Harris et al., '44; Johnson and Baumann, '48). It is possible that the slight superiority of alfalfa over carotene, with respect to utilization as vitamin A, was due to the tocopherol content of the alfalfa. However, the tocopherol content of the basal ration was calculated to supply approximately two and one-half times the requirement of the chicken. Further work is required to clarify this point.

Each egg laid by the three birds in each pen was analyzed for vitamin A and carotene and the values averaged for weekly

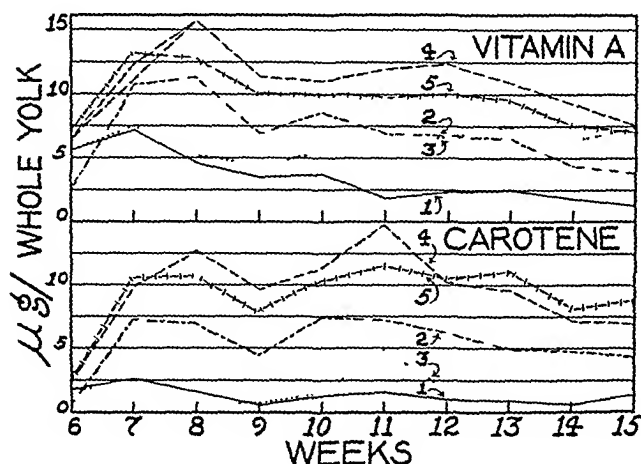


Fig. 3 Vitamin A and carotene content of egg yolk. 1. Basal ration. 2. Carotene supplement. 3. Vitamin A supplement. 4. Fresh alfalfa supplement. 5. Dehydrated alfalfa meal supplement.

periods, as is shown in figure 3. The values are given for the last 70 days of the experimental feeding period.

An analysis of variance showed highly significant differences between pens for the vitamin A and carotene content of egg yolk. The minimum difference required for significance (M. S. D.) between pens, for the vitamin A content of egg yolk, was 1.118 at the 0.01 level and 0.849 at the 0.05 level. For the carotene content of egg yolk the M. S. D. was 1.727 at the 0.01 level and 1.312 at the 0.05 level.

As is shown in figures 3 and 4, the carotene content of the egg yolk had been reduced to a low level at the end of the 33-

day depletion feeding period. The vitamin A content remained at a relatively higher level. On the 10th day of the experimental feeding period the first visible carotenoid pigments appeared in the petroleum ether extract of the egg yolk from the birds receiving alfalfa supplements. This first color was removed in the phasic separation of carotene, indicating the pigments to be carotenoids other than carotene. The carotene content of the egg yolk from the negative control pen, 1, gradually decreased during the 72-day experimental feeding period. The carotene content of the egg yolks from the birds receiving vitamin A was low, as would be expected.

Analysis of variance shows that, for the vitamin A content of egg yolk at the 0.01 level, pens 1, 2, and 3 each differ significantly from all the other pens. Pen 4 differs significantly from pen 5 at the 0.05 level. It will be noticed, in figure 3, that the eggs from pen 3 show a gradual increase in vitamin A content, while the general trend in the eggs from the other 4 pens is a decrease in vitamin A content. The vitamin A content of the eggs from pen 3 did not differ significantly from that of eggs from pens 4 and 5 at the termination of the experiment. The carotene content of the egg yolks, at the 0.01 level, from pens 1 and 3 each differs significantly from that of yolks from pens 2, 4, and 5, and pen 2 differs significantly from all the other pens.

The above differences in the vitamin A and carotene content of egg yolk further indicate that  $\beta$ -carotene in cottonseed oil is utilized less efficiently than are the vitamin A-active substances found in alfalfa. Further study is necessary to determine more accurately the extent of utilization of carotene as found in fresh alfalfa and dehydrated alfalfa meal. It is of interest to note that vitamin A from fish liver oil caused a definite increase in the liver reserves of vitamin A as contrasted to that from fresh alfalfa and dehydrated alfalfa meal, yet the alfalfa supported the higher egg yolk content of vitamin A. Furthermore, if all the carotene in the egg yolks from the birds receiving alfalfa is converted to vitamin A, the vitamin A value of the eggs would be approximately twice that of the eggs of

birds receiving carotene in oil, and three times that of the eggs from birds receiving vitamin A. It thus appears that alfalfa is more effectively utilized by chickens as a source of vitamin A-active substances in eggs than is vitamin A from fish liver oil, whereas the latter permits greatest liver storage.

Figure 4 shows the production record for the 105 days of the experiment.

There is normally a steady rise in egg production during the period in which the experiment was conducted. It will be noticed that at about 9 weeks there was a definite break in egg

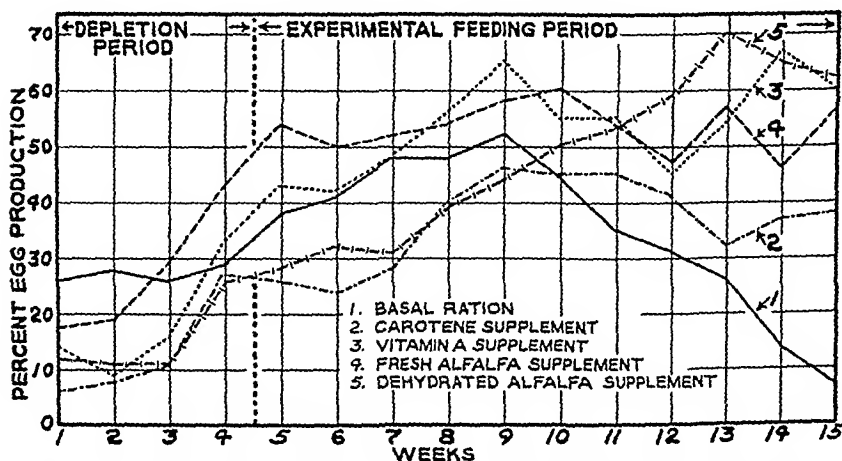


Fig. 4 Per cent egg production during the experimental period of 105 days.

production in the negative control pen, 1, and some indication of a break in pen 2. During the last week of the experiment egg production was 7% in pen 1, 38% in pen 2, 60% in pen 3, 56% in pen 4, and 62% in pen 5. The difference in egg production between pen 1 and pens 3, 4, and 5 appears to be significant. The difference between pen 2 and pens 3, 4, and 5 is indicative of significance. The sudden break in production at about 9 weeks supports the conclusions of McClymont and Hart ('48), who found that inadequate intake of vitamin A in white Leghorn pullets resulted in cessation of egg production at about 120 days, with death resulting at about 165 days. The

rate of egg production was affected only slightly before total cessation of production.

The syndrome of avitaminosis A was first observed at about the time of the break in egg production (9 weeks). The birds were droopy, refused food and water, and were in a badly emaciated condition. Multiple gray-colored retention cysts, each 1 to 2 mm in diameter, were found in the mucosa of the oesophagus and hard palate of most birds in pen 1. Enlarged ureters and kidneys filled with urates, a condition commonly associated with avitaminosis A in chickens, were not found as consistently as were the cysts in the oesophagus. The above conditions, together with sudden mortality, accounted for the termination of the experiment on May 18, 1948.

#### SUMMARY

Liver reserves of vitamin A in laying pullets partially depleted of their vitamin A and carotene reserves continued to decline when carotene equivalent to 2,000 I.U. of vitamin A activity per pound of ration was supplied in the form of carotene in cottonseed oil, fresh alfalfa, and dehydrated alfalfa meal. The carotene in oil was utilized less efficiently than that in the alfalfas. The carotene in fresh alfalfa was utilized somewhat more efficiently than that in dehydrated alfalfa meal. Vitamin A from fish liver oil, fed at the same level of vitamin A activity, enabled laying pullets to increase gradually their reserves of vitamin A in the liver.

The amounts of vitamin A and carotene in the eggs laid were greatest from pullets receiving the alfalfa supplements, while the next highest were from pullets receiving carotene in oil.

The total potential vitamin A activity in eggs from the pullets receiving the alfalfa supplements was about twice that of the eggs from birds receiving carotene in oil and three times that of birds receiving vitamin A from fish liver oil. Thus, vitamin A was superior in building liver reserves of the vitamin, while carotene from alfalfa, fresh or dehydrated, was

superior in transfer of vitamin A and carotene to the egg. Alfalfa appears to carry a factor which enhances the utilization of carotene.

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# THE RELATIONSHIP OF DIETARY FACTORS TO THE TOXICITY OF ALLOXAN<sup>1</sup>

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It has been reported by Houssay and Martinez ('47) that dietary factors may influence the response of rats to alloxan. Mortality within 7 days following the injection was used as the criterion of the sensitivity of the animals. No data on blood sugar were presented but it was stated that in all cases marked hyperglycemia preceded death. Using as a base line a diet high in carbohydrate and low in fat, containing 20% protein, low protein or high lard or ox-fat caused increased mortality. Methionine or thiouracil (but not choline) supplementing the high lard diet overcame the deleterious effect of the fat. Animals receiving diets high in butter or olive oil responded in the same fashion as those on the low fat diet, while high oleomargarine, corn oil, or cocoanut oil had some beneficial effect. There is a similarity between the factors involved here and those which influence the development of massive hepatic necrosis (György, '44; Himsworth, '47). Of the group of substances which protect the liver against necrosis, only tocopherol (Schwartz, '44a, b; György, '47) is not mentioned. The present work was undertaken primarily to determine whether tocopherol might also protect the pancreas against the necrotic action of alloxan.

<sup>1</sup> Supported by a grant from Swift and Company, Chicago.

A major finding of this study, the extensive intravascular hemolysis and consequent high mortality within one or two days following the administration of alloxan to tocopherol-deficient rats, has been reported (György and Rose, '48). Further investigations in connection with this problem have been briefly presented (Rose and György, '49) and will be discussed in more detail in a later communication. The present report is concerned with the effect of tocopherol, as well as of some of the other dietary factors investigated by Houssay and Martinez, on the development of diabetes and on survival following alloxan injection.

#### EXPERIMENTAL

The general plan of the experiment followed that of Houssay and Martinez. Young female rats, weighing 90 to 135 gm, were given the experimental rations for about one month. At the end of this time they were fasted overnight and injected with 160 mg per kilogram of alloxan. Food intake was measured throughout the experimental period.

In the first study the animals were obtained from a local dealer. In all succeeding experiments the rats were of the Sprague-Dawley strain.

The experimental rations were of the type which had previously been used in producing hepatic injury, consisting insofar as possible of highly purified ingredients (table 1). The vitamin B complex was not incorporated in the diets but the crystalline vitamins (thiamine chloride, 20  $\mu$ g; riboflavin, 25  $\mu$ g; pyridoxine, 20  $\mu$ g; and calcium pantothenate, 100  $\mu$ g) were given daily as a separate supplement. Houssay and Martinez had used wheat and corn flours as the source of carbohydrate and yeast to supply vitamin B.

The experiments may be divided into three series. In the first experiment the dietary protein was low, as in studies of liver injury; in the second the protein level was raised to 20%, while in the third the 20% protein diets were modified by the addition of 5% of yeast, the regular vitamin B supplement not

being discontinued. A single group of animals in the second experiment was given a diet containing 40% of casein; a similar group in the third experiment received high protein and yeast (table 1).

TABLE 1  
*Composition of diets*

DIET TYPE	CASEIN <sup>1</sup>	SUCROSE	FAT	COD LIVER OIL	YEAST	SALT MIXTURE
	%	%	%	%	%	%
Low protein, high fat	8	48	38	2		4
Low protein, low fat	8	88	"	"		4
Normal protein, high fat	20	36	38	2		4
Normal protein, high fat with yeast	20	31	38	2	5	4
Normal protein, low fat	20	76	"	"		4
Normal protein, low fat with yeast	20	71	"	"	5	4
Normal protein, high fat, no cod liver oil	20	76	40	"		4
High protein, high fat	40	16	38	2		4
High protein, high fat with yeast	40	11	38	2	5	4

<sup>1</sup> Vitamin test casein, General Biochemicals, Inc.

<sup>2</sup> Supplemented with three drops of corn oil daily.

<sup>3</sup> Supplemented with three drops of Mead percomorph oil weekly.

Three fats were studied: lard, hydrogenated cottonseed oil<sup>2</sup> and cocoanut oil. The high fat diets contained, in addition to 38% of the experimental fat, 2% of cod liver oil. The low fat ration contained no fat in the diet mixture, the animals being given supplements of three drops daily of corn oil to supply essential fatty acids and three drops weekly of percomorph oil for vitamins A and D. One group of animals on the high lard ration was given percomorph oil instead of cod liver oil to determine whether any benefit resulting from the low fat diet might be due to this substitution rather than the low level of dietary fat.

<sup>2</sup> "Vream," Swift and Company.

Methionine <sup>3</sup> (50 mg per day) and choline (25 mg per day) were fed with the vitamin B supplement. Tocopherol,<sup>4</sup> in the form of mixed natural tocopherols, was given either as a separate supplement of 3 mg per day dissolved in one drop of cottonseed oil or incorporated in the diet at a level of 50 mg per 100 gm to give approximately the same daily dose.

Alloxan <sup>5</sup> (160 mg per kilogram) was injected intraperitoneally, a freshly prepared solution containing 10 or 16 mg per milliliter being used. It was not possible to handle all of the animals of an experiment simultaneously, so one or two rats from each group were injected on the same day.

Blood sugar determinations <sup>6</sup> were made 48 hours after alloxan administration on all surviving rats, with a few exceptions where the condition of the animals made it impossible to obtain a blood sample. The animals were not fasted. Blood non-protein nitrogen (NPN) was determined at the same time on all animals of experiment II and half of those of experiment III. Sugar was estimated by the method of Somogyi ('37, '45) as modified by Nelson ('44), and NPN by an adaptation of the method of Folin and Denis ('16). Blood for both of these determinations was obtained from the tail.

The studies were first carried out with 10 rats in each group. All three experiments were then repeated so that most of the groups finally included 20 animals. In the second and third experiments there was no experimental difference between the two sections, so the values on the 20 animals of each group could be averaged together. In those groups which received the low protein diets (experiment I) there was a difference in the source of the rats in the two parts of the experiment. The very marked difference in the results of the two series required their separate consideration.

<sup>3</sup> Kindly furnished by Wyeth, Inc., Philadelphia.

<sup>4</sup> Kindly furnished by Distillation Products, Inc., Rochester, N. Y.

<sup>5</sup> Eastman Kodak Company.

<sup>6</sup> The blood sugar determination were made in the Cox Institute through the courtesy of Dr. F. D. W. Lukens.

TABLE 2  
Effect of dietary factors on alloxan toxicity

GROUP	NO. OF RATS	BODY WEIGHT		BLOOD SUGAR					MORTALITY		FOOD INTAKE	
		Start	Change	No. of def'n's.	Aver age	No. of def'n's. 200 mg %	mg %	mg %	2 days	After 7 days	Before alloxan	After alloxan
gm	%	Experiment I a: Low protein	Experiment II "Normal" protein	Experiment III "Normal" protein + yeast	%	%	gm/day	gm/day				
High hard	10	120	-21	0	400	0	20	100	4.8	2.2		
High hard + methionine	10	120	-18	10	365	1	0	60	6.1	2.0		
High hard + tocopherol	10	120	-17	8	400	0	20	100	5.2	2.0		
High Vitam	9	122	-17	8	390	1	0	67	5.5	2.5		
High coconut oil	10	121	-20	10	355	3	0	70	5.5	2.8		
High hard	10	119	-18	0			100		5.1			
High hard + methionine	10	118	-8	0			100		5.1			
High hard + tocopherol	10	119	-22	0	500	0	60	90	4.8	1.4		
High Vitam	9	119	-18	7	410	2	0	67	5.6	2.2		
High coconut oil	9	122	-18	2	440	0	90	100	5.2	1.1		
Low fat	10	118	-8	4	305	2	30	50	8.1	7.6		
Low fat + tocopherol	10	121	-9	5	355	2	30	60	8.7	4.7		
High hard	20	110	+6	10	485	0	55	95	5.0	4.9		
High hard + methionine	20	108	+43	10	370	1	50	100	5.8	1.2		
High hard + choline	20	108	+36	12	410	0	35	75	5.9	2.9		
High hard + tocopherol	20	109	+15	16	390	0	25	85	5.3	2.1		
High Vitam	20	108	+18	6	475	0	31	100	4.3	1.7		
High coconut oil	20	109	+25	18	510	2	10	65	5.7	2.8		
High coconut oil + tocoph. <sup>1</sup>	20	109	+2	0	280	1	175	80	4.9	2.1		
High hard without cod liver oil	10	106	-1	7	330	2	20	80	3.7	1.5		
Low fat	10	112	+12	2	430	0	80	100	5.5	2.1		
Low fat + tocopherol	20	108	+23	14	660	2	25	55	8.0	3.2		
Low fat + high protein	20	106	+26	18	520	1	105	30	7.7	6.3		
High hard + high protein	20	108	+24	5	400	0	75	95	4.9	1.7		
High hard	20	107	+41	17	560	1	15	90	6.3	1.6		
High hard + methionine	20	106	+57	15	550	1	35	80	6.5	2.3		
High hard + choline	20	107	+48	11	450	2	35	85	6.8	1.8		
High hard + tocopherol	20	109	+47	20	455	0	95	90	6.5	2.5		
High hard + tocopherol + methionine	20	108	+49	17	500	1	15	85	6.7	1.1		
High Vitam	20	108	+64	20	540	1	90	75	7.4	3.6		
High coconut oil	20	108	+53	19	700	1	170	95	6.7	2.1		
High coconut oil + tocopherol <sup>2</sup>	20	108	+35	18	310	6	85	55	6.1	2.2		
Low fat	20	107	+36	19	385	1	115	55	6.1	2.0		
Low fat + tocopherol	20	108	+47	18	515	2	6	20	10.7	5.1		
Low fat + high protein	20	107	+52	18	610	2	130	35	10.3	6.1		
High fat + high protein	20	108	+58	17	435	3	115	95	6.3	2.1		

<sup>1</sup> Average NPN before treatment with alloxan = 50 mg %.<sup>2</sup> Average intake for two to 4 days following alloxan injection in rats with diabetes.<sup>3</sup> Tocopherol mixed with the dietary fat.<sup>4</sup> In experiment III the NPN was measured on half of the animals of each group.

In our experiments the proportion of the rats dying within 7 days after injection with alloxan was not an entirely satisfactory criterion for estimation of the influence of the dietary factors studied. It made no distinction between the groups in which the majority of the animals died during the first day or two after the injection and those in which the deaths were spread over 5 or 6 days. Since this difference proved to be of significance, mortality within two days after the administration of alloxan as well as during the first week has been tabulated.

#### RESULTS

Tocopherol was found to be the most important factor involved in preventing early mortality. The effect of tocopherol was the same whether it was fed as a separate supplement or was mixed with the lard or cocoanut oil of the diet. The same favorable response was observed in animals which received diets containing an hydrogenated cottonseed oil of which tocopherol is a natural constituent. An explanation of the high early mortality of the tocopherol-deficient animals was found in the hemolysis which has been described previously (György and Rose, '48). Hemolysis was never observed in any animals treated with tocopherol. This all-or-none response could not be duplicated in the death rate, since the toxicity of alloxan was not due solely to hemolysis. In most of the individual groups the mortality of the animals treated with tocopherol was definitely lower than that of those deficient in tocopherol, and when larger units were considered the differences were unquestionable. So, in the series receiving the diets containing 40% of fat and 20% of casein, mortality during the first two days after injection in all of the tocopherol-deficient groups combined was 51%, in the tocopherol-treated groups, 16%. In only one experiment (Ia) was the mortality in the groups which had not received tocopherol low during this initial two-day period. These animals were the only ones of the whole series which were not obtained from Sprague-Dawley, and they may have received enough tocopherol during the pre-experimental

period to protect them against depletion during the month on the tocopherol-deficient diet.

The protective effect of tocopherol seemed to be limited to the period immediately following administration of alloxan. At the end of a week the tocopherol-treated groups had almost overtaken the deficient ones in the number of deaths. In the series referred to above, namely the groups receiving diets high in fat and with normal content of casein, mortality of the tocopherol-deficient animals within 7 days was 87%; of the tocopherol-treated ones, 79%.

Yeast showed an effect on survival similar to but less pronounced than that of tocopherol. When 5% of yeast was added to the high fat rations, mortality was reduced from 51% to 25% during the first two days, but after 7 days 79% of the animals were dead. Tocopherol with yeast lowered the death rate during the early period to 8%, but even with this combination 74% of the animals died within one week.

Variation of the protein content of the diet had little effect on survival. Sprague-Dawley rats receiving only 8% of casein were somewhat more susceptible to the effects of a deficiency of tocopherol immediately after injection with alloxan than those on the diet containing 20% of casein. When the 7-day period was considered, however, there was as high mortality with 20% and even with 40% of casein as with 8% in the groups which had received diets with a high content of fat. With low-fat diets there was a slight, but statistically insignificant, difference in favor of a normal over a low level of protein.

Methionine and choline, substances which have a specific protective effect on the liver, were quite without influence on the toxicity of alloxan. Mortality during the two-day and the 7-day period was as high as with the unsupplemented diet. Methionine in conjunction with tocopherol gave no advantage over tocopherol alone.

Few rats receiving high fat diets survived more than 7 days. Mortality was low during the first two days when the hydro-

generated cottonseed oil was used, because it contained tocopherol. There was a slight advantage shown by cocoanut oil over lard or cottonseed oil at the end of 7 days in those groups which had received yeast, but not in those without yeast.

When fat was eliminated from the diet the toxicity of alloxan was much reduced. Only with this type of diet did an appreciable proportion of the rats survive more than 7 days after the injection. The average survival for this period of all the animals on the low fat diets was 61%, while the average of all the high fat groups was 19%.

Most of the animals injected with alloxan became diabetic regardless of the nature of the diet which they had received. The average level of blood sugar 48 hours after alloxan was given was high in all groups, ranging from 280 to 700 mg per 100 ml. In view of the wide range of the individual values, no statistical significance can be assigned to the group averages. Arbitrarily designating a blood sugar level exceeding 200 mg per 100 ml as an indication of the presence of diabetes, a slightly lower incidence of the disease was observed in animals receiving diets low in fat or containing cocoanut oil. With high lard or high cottonseed oil content, 94% of the animals surviving more than 48 hours had diabetes; with low fat diets, 86%; and with high cocoanut oil diets, 81%. These differences, though small, are significant ( $P < 0.001$ ).

The general picture in almost all animals which survived more than 7 days was a gradual improvement in the diabetes. Blood sugar values were lower after several weeks, and fasting sugar values almost normal. Most of the animals showed persistent moderate to severe glycosuria. Polyuria and glycosuria were especially prominent in the rats receiving the low fat (high carbohydrate) diet.

Blood non-protein nitrogen, measured 48 hours after injection of the animals, was elevated in almost all cases (György and Rose, '49), the highest values being found in the tocopherol-deficient groups. The kidneys of most of the animals were examined histologically and the same type of damage



found in tocopherol-treated and tocopherol-deficient groups.<sup>7</sup> The higher NPN of the tocopherol-deficient animals was probably a consequence of the blocking of the kidneys with hemoglobin from the damaged red blood cells.

#### DISCUSSION

Under the conditions of our experiments the dietary factors studied seemed to be of little significance with respect to the diabetogenic effect of alloxan. The premise that there are similar protective agents effective against necrosis of the pancreas and liver was not sustained. Methionine, choline and tocopherol were equally ineffective in preventing elevation of the blood sugar level following alloxan administration. Elimination of fat from the diet resulted in only a small reduction in the incidence of diabetes. There was a slightly greater effect when coconut oil was introduced into the diet, but even in this case 81% of the animals were diabetic.

The significance of the blood sugar values as an indication of diabetes may be lessened to some extent by two factors, kidney damage and food intake. That the former was without appreciable effect may be seen by the similarity of the average figures for blood sugar in the tocopherol-deficient groups, where nitrogen retention was generally large, and in the tocopherol-treated groups, which showed only moderate retention. Food intake after the injection was low in all groups but somewhat higher in those given the low fat than in those receiving the high fat rations. The elevation of the blood sugar values may have been exaggerated somewhat in the low fat groups, but the persistent excretion of large volumes of urine with a high content of sugar gave additional evidence of severe damage to the pancreas.

<sup>7</sup> Microscopic examination of a large number of kidneys and livers obtained from our animals revealed severe acute tubular changes in the kidneys and often intensive parenchymatous (hydropic) degeneration in the liver. There was no indication of fatty degeneration, and glycogen stains showed, if anything, less than a normal content of glycogen. We are indebted to Dr. Harry Goldblatt, Institute for Medical Research, Los Angeles, for these findings.

Mortality within 7 days was the criterion used by Houssay and Martinez ('47) in determining the severity of the effect of alloxan. We were unable to duplicate their finding that methionine would reduce mortality after alloxan administration in rats receiving a diet high in lard. Even with 50 mg per day of methionine most of the animals died within 7 days. We did observe much better survival with low fat than with high fat diets. Houssay and Martinez found replacement of lard with cocoanut oil even more effective than its replacement with carbohydrate, mortality being reduced from 100% to 0%. In our experiments, in only two of the 6 pairs of groups differing only as to whether they were fed lard or cocoanut oil as the dietary fat was there a significant reduction in the number of deaths. Considering the 6 pairs collectively the difference is of definite mathematical significance, though of less importance practically since mortality during the first week was still 69% as compared with 93% for the animals receiving lard and 35% for those on the low fat diet.

Although recent interest in alloxan has been concentrated on its ability to produce diabetes, many other toxic effects have been reported. Its action as a capillary poison and spasmodic was studied by Labes and Freisburger ('30) and the severe kidney damage following its injection has been described many times (compare Lukens, '48). In the present paper we have discussed the hemolyzing effect of alloxan in tocopherol-deficient rats. In our experiments diabetes did not seem to be primarily responsible for the death of the animals. Mortality and diabetes were decreased to about the same extent when cocoanut oil was fed, but the effect was too small to allow any conclusions to be drawn from it. Only rats receiving diets low in fat had an appreciably prolonged survival, and in most of these diabetes, as measured by blood and urine sugars, was severe. Nor, in these animals, was nitrogen retention less than in other groups. The response of some other tissues to the action of alloxan must be modified by the feeding of fat or carbohydrate.

An explanation has been offered for the protective effect of tocopherol (György and Rose, '49). In its absence, either blocking of the kidneys with hemoglobin or anemia may be in part responsible for the death of the animal, or may weaken its resistance to the other toxic effects of alloxan. It has been found that, as far as hemolysis is concerned, tocopherol acts specifically in the red blood cell, presumably through its anti-oxidant activity (Rose and György, '49). The mode of action of yeast is not so definite. Animals receiving yeast were better nourished than those without it, but the greater effectiveness of yeast with respect to survival in the absence of tocopherol than when fed as a supplement to it, and the failure of protection after 7 days, suggest that its action may be similar to that of tocopherol. Hemolysis was not prevented completely by yeast as it was by tocopherol, but it was reduced in incidence and severity. Yeast contains anti-oxidants (György and Tomarelli, '43) and these may be effective in reducing the toxicity of alloxan.

The two experiments with the low protein diet, in which rats obtained from different sources were used, show how much care must be used in the interpretation and general application of the results of experiments of this type, particularly in studies of such short duration. In the first study there was almost no mortality in the tocopherol-deficient groups during the first two days after alloxan administration; in the second, very high mortality figures were recorded. The probable explanation of this discrepancy is a difference in tocopherol intake during the pre-experimental period. The data indicate that with the first rats used the reserve of tocopherol was sufficient that the animals were not depleted by one month on the tocopherol-deficient diet, and consequently neither the very severe symptoms of the latter group nor any benefit of tocopherol were observed.

#### SUMMARY

The influence of dietary factors on the sensitivity of rats to alloxan has been investigated. The diet was varied with re-

spect to its content of protein and fat, and supplements of methionine, choline, tocopherol, and yeast were given.

The toxicity of alloxan, as measured by the number of rats which survived for 7 days after its injection, was decreased appreciably only when diets very low in fat were fed. There was a slight advantage shown by cocoanut oil over lard or hydrogenated cottonseed oil in the high fat diets. The hemolysis which occurred when alloxan was administered to tocopherol-deficient rats caused a high death rate during the first two days after the injection. Tocopherol and, in an apparently somewhat similar fashion, yeast, reduced the early mortality but had no effect after this initial period.

Diabetes developed in most of the animals regardless of the nature of the diet. Considering those animals as diabetic whose blood sugar, 48 hours after injection with alloxan, was above 200 mg per 100 ml, the incidence of diabetes was slightly, but significantly, lower with diets low in fat or containing cocoanut oil than with those containing lard or hydrogenated cottonseed oil. There was no evidence of any benefit from any other of the variations in diet studied. Even tocopherol, in spite of its very specific effect in preventing hemolysis following the injection of alloxan, gave no protection against the diabetogenic effect of alloxan.

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# THE VITAMIN A REQUIREMENT OF GROWING TURKEYS<sup>1</sup>

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THREE FIGURES

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Investigations of the vitamin A requirement of growing turkeys have produced diverse results. Scott and Hughes ('32), using a diet containing white corn, ground wheat, wheat bran and meat and bone scraps with yellow corn as the source of vitamin A, reported that 50 or 60% of the ration should consist of yellow corn to provide the minimum requirement of vitamin A for growing turkeys. This amounts to approximately 350 to 420 I.U. of vitamin A per 100 gm of feed. Hinshaw and Lloyd ('34) reported 1,736 I.U. of vitamin A per 100 gm of feed as the minimum requirement. Their diet contained white corn, barley, wheat, fish scrap, dried milk, bone meal, ground limestone and salt, with alfalfa leaf meal as the source of vitamin A. From a nutritional standpoint this diet is an improvement over the one used by Scott, yet, fortified with alfalfa leaf meal at the level reported as optimum it produced birds averaging only 1,200 gm approximately at 12 weeks of age. Wilgus ('40), using a more complete diet

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including soybean oil meal, casein, dried brewers' yeast, manganese sulfate and irradiated sterol, with crystalline carotene as the source of vitamin A, reported 600 I.U. of vitamin A per 100 gm of feed as meeting the requirements of growing turkeys. Weights at 8 weeks of age at this level averaged 1,033 gm. Scott, Matterson and Jungherr ('45-'46) obtained average weights of 1,376 gm at 8 weeks of age on a vitamin A deficient diet fortified with 1,000 I.U. of vitamin A per 100 gm of feed, and concluded that this level of vitamin A was optimum. The National Research Council ('44) recommended an allowance of 551 I.U. per 100 gm of feed.

A serious fault with previously conducted investigations to determine the vitamin A requirement of growing turkeys has been the poor growth obtained on the diets used. A casual examination of most of these diets clearly reveals that many were deficient in the B complex vitamins. It appears that factors other than vitamin A limited the growth in most, if not all, of these experiments.

#### EXPERIMENTAL

The diet used in the experiments reported herein (table 1) was an improved modification of the 1946 Connecticut vitamin A "free" turkey diet. The experimental diet was supplemented with all the known crystalline B vitamins at or above the level recommended by the National Research Council ('44, '46). Where requirements have not been determined, the vitamins were added at a level indicated as sufficient by previous experiments conducted at the Storrs Agricultural Experiment Station (unpublished data). This diet, properly fortified with vitamin A, produced poults averaging over 2,000 gm at 8 weeks of age.

A basal diet containing all the ingredients except white corn was mixed in amounts deemed sufficient to last for the duration of each experiment, although in one case it was necessary to mix an additional amount.



A premix of white table corn meal and cod liver oil<sup>5</sup> assayed at 2,225 I.U. of vitamin A per gram was substituted for white table corn meal in the basal ration to provide experimental diets containing 0, 150, 300, 600, 1,200, 2,400, 4,800 and 7,200 I.U. of vitamin A per 100 gm of feed for lots 1 through 8, respectively. The premix and individual lot diets were mixed at intervals of 10 days and stored at  $-10^{\circ}\text{C}$ .

In the first two experiments 161 unsexed day-old Broad Breasted Bronze poults from the college flock were used. These poults were divided into weight groups covering a

TABLE 1  
*Composition of the vitamin A "free" ration*

INGREDIENT	AMOUNT	INGREDIENT	AMOUNT
	lb.	<i>Vitamin supplement/100 lb.</i>	
White table corn meal	53.4	Choline chloride	104.00 gm
Acid precipitated casein	20.0	Nicotinic acid	2.27 gm
Soybean oil meal	15.0	2 Methyl-naphthoquinone	45.00 mg
Butyl fermentation solubles <sup>1</sup>	4.0	Riboflavin	227.00 mg
Wheat germ oil	2.0	Ca pantothenate	635.60 mg
Salt	0.5	Thiamine	90.80 mg
Ground limestone	1.0	Inositol	227.00 mg
Bone meal	4.0	Para-aminobenzoic acid	605.00 mg
Activated animal sterol		Pyridoxine	227.00 mg
200 D/gm	0.1		
MnSO <sub>4</sub>	0.025		

<sup>1</sup> Curbay B G "80."

range of 5 gm. Equal numbers of poults from each weight group were selected at random to make up the necessary lots. The poults were wing banded and weighed and individual weights were taken weekly for the duration of the experiment.

The 188 poults used in experiment 3 were male day-old Broad Breasted Bronze poults obtained from a commercial hatchery. They were divided into lots by the method described above and the experiment was replicated three times. Birds in replicate 3 were used for laboratory tests.

All poults were kept in a standard Petersime electric battery brooder and at 4 weeks of age were moved to new locations

<sup>5</sup> Kindly supplied by Dr. John Correll of the Upjohn Company, Kalamazoo, Michigan.

in standard developing batteries. Replicated experiments were arbitrarily assigned a battery for each replicate and the location of each lot was chosen at random.

Two birds from each lot, experiment 3, replicate 3, were sacrificed biweekly, beginning at the second week, for vitamin A and carotene determinations on blood and liver.

The method of Kimble ('39) was essentially followed in extracting vitamin A and carotene from the blood samples. The saponification of liver samples and the extraction of vitamin A followed the method of Davies ('33). The activated 1-3 glycerol dichlorohydrin method of Sobol and Werbin ('45) was used for the determination of vitamin A.

Blood samples were secured by bleeding from the heart and determinations were made the same day the samples were drawn. The liver samples were refrigerated under potassium hydroxide until the analysis could be made. Beak and brain sections were taken for histological study.<sup>6</sup>

Experiment 1 was started May 3 and contained 7 lots, replicated twice. This experiment was closed when the birds were 6 weeks of age. Experiment 2, a duplicate of experiment 1, was started May 17. The poults in experiment 3, started June 24, were divided into three replicates. A negative control (lot 1) was established in replicate 3 only and an additional level of vitamin A (lot 8) included. Experiments 2 and 3 were carried until the poults were 8 weeks of age.

At the close of each experiment the sex of each bird was established. An incision was made in the dorsal region between the 6th and 7th rib, exposing the abdominal cavity, and the sex was determined on the basis of the presence or absence of a testicle.

All birds were allowed ad libitum feeding and feed consumption records were kept.

The growth data analysis was based on a modification of the Wishart ('39) method. The basic principal in this method involves analysis based on constants used in fitting the weight

<sup>6</sup> Histological interpretations were made by Dr. E. Jungherr of the Animal Disease Laboratory, Storrs Agricultural Experiment Station.

observations of each animal to a curve. This method allows the use of the original data, and differences in the response over the entire experimental period can be measured.<sup>7</sup>

The logarithms of the weights were used in this analysis since, according to Wishart, growth differences can be elucidated by examination of one or two constants of the curves fitted to the logarithm of the weights.

Two terms, a linear and a quadratic, were used to fit the logarithm of the weights of the male poults to a curve. Graphical examination of these coefficients revealed that the linear coefficient, representing the slope of the curve, was related to dosage of vitamin A; and that the quadratic coefficient, descriptive of the departure from linearity, was not related to treatment. Subsequent analyses were then made on the linear coefficient.

The mean, adjusted coefficients were fitted to a curve by the method of covariance with two concomitant measures, and plotted against log-dose. This method allows the number of individuals in each lot to influence the derived or calculated point on the curve it will occupy, and enables the calculation of the point of greatest response to treatment if the range of treatment is sufficient to reach a point of plateau of diminished response.

## RESULTS

### *Growth*

Figure 1 shows the adjusted mean linear coefficients plotted against the log-dose as the abscissa. The response to increased levels of vitamin A up to 6 weeks of age (expt. 1) can be compared with the response up to 8 weeks of age in experiments 2 and 3. The calculated point of greatest response is at levels of 1,760, 1,512, and 1,776 I.U. of vitamin A per 100 gm of feed for experiments 1, 2 and 3, respectively.

The negative controls in all experiments died by the end of the 4th week. All were found to have gross evidence of

<sup>7</sup> Details of the statistical treatment of lots will be published in expanded form in a forthcoming Experiment Station bulletin.

A-avitaminosis. While ataxia was a fairly constant observation, many birds died suddenly without evidencing any gross pathological symptoms.

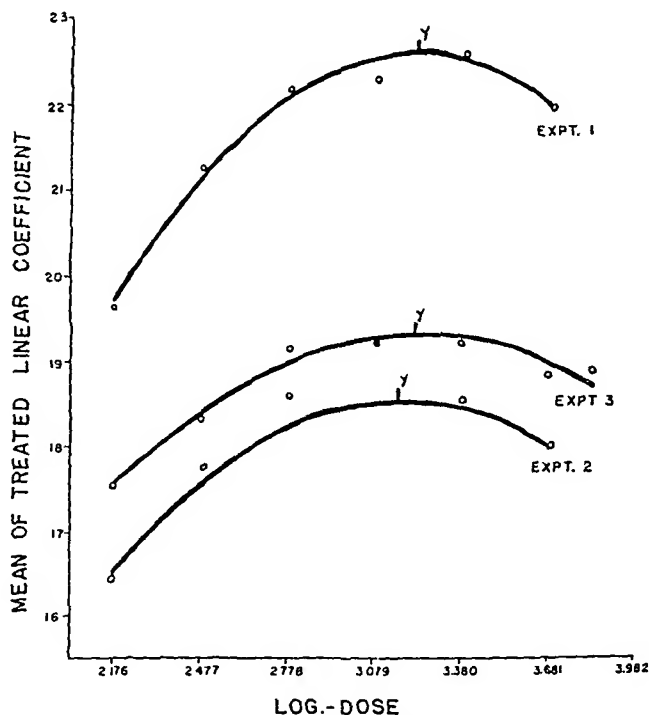


Fig. 1 Average linear coefficients for experiments 1, 2 and 3 plotted against concentration of vitamin A in the diet together with calculated point of greatest response.

### *Blood levels of vitamin A*

A rapid rise in the concentration of vitamin A in the blood with increases of vitamin A in the diet to the 1,200 I.U. per 100 gm of feed level is shown by the 6-week data, which is plotted graphically in figure 2. Beyond this point blood vitamin A concentration more or less reaches a plateau.

### *Liver storage of vitamin A*

In all lots receiving less than 1,200 I. U. of vitamin A per 100 gm of feed, liver stores of vitamin A decreased between

the second and 4th week. At this point lots 2 and 3 reach a plateau and lot 4 shows a slight increase in storage (fig. 3). Liver storage at two weeks of age was significantly higher in the birds of lots 5, 6, 7 and 8. Lot 5 (1,200 I. U.) maintained its liver stores over the experimental period, and lots 6, 7 and 8 substantially increased their liver stores with age.

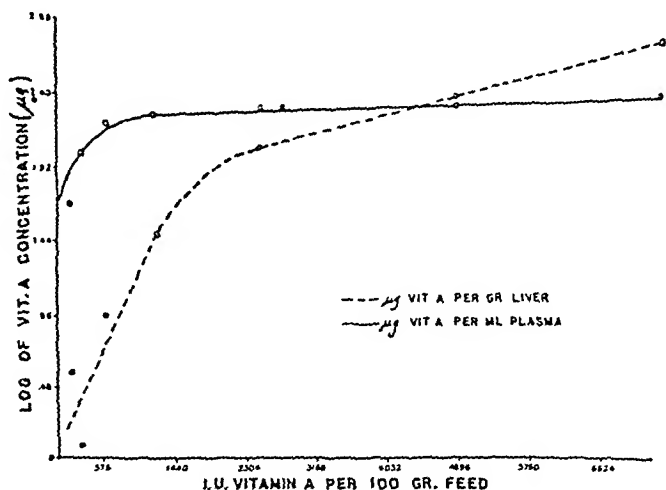


Fig. 2 Concentration of vitamin A in blood plasma and liver for poults at 6 weeks of age on increasing levels of vitamin A in the feed.

### *Histopathology*

No nasal lesions were observed in any bird receiving 150 I.U. of vitamin A per 100 gm of feed or amounts above this, nor were lesions found in any birds at two weeks of age. Of the 9 poults started in the negative control group, only one survived to 4 weeks of age and in this bird only were the characteristic lesions of A-avitaminosis found.

Lesion in the brain stem and optic chiasma which, according to Adamstone ('47), are specific for vitamin A deficiency in chickens, occurred in turkeys 6 and 8 weeks of age on all levels of vitamin A. No lesions were found in any bird under 6 weeks of age receiving a vitamin A supplement.

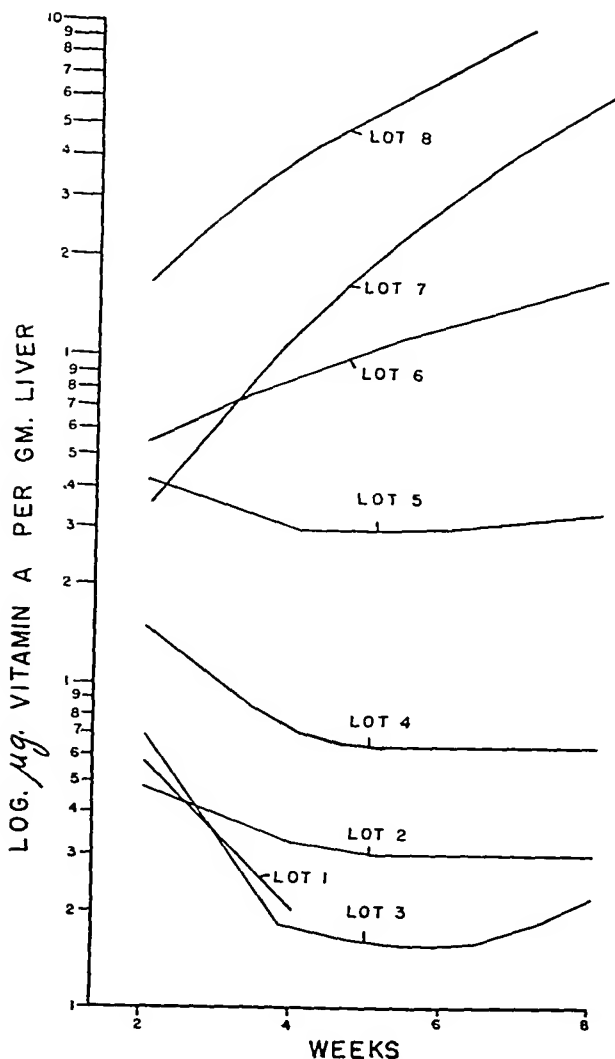


Fig. 3 Liver storage of vitamin A in poult on increasing levels of vitamin A in the diet plotted against time.

#### DISCUSSION AND CONCLUSIONS

There is evidence to indicate that males have a higher vitamin A requirement than females (Scalongne, '40; Brenner, Banks and Roberts, '42; Popper and Brenner, '42). On this premise it was reasoned that data secured from the male poult

would represent the most valid basis for determining the vitamin A requirement of the turkey poult. Therefore only the male birds in the three experiments reported herein were used as a source of growth data.

The use of growth as a criterion for the measurement of the vitamin requirement of an animal does not preclude nor invalidate the use of such criteria as longevity, lactation, egg production, et cetera. It does, however, assume that maximum growth is optimum growth; thus the minimum requirement for any nutrient for growth may be defined as the lowest level of that nutrient which allows the maximum growth inherently possible. This definition describes equally well the maximum requirement because, according to it, additional increments of the nutrient fail to induce additional response in growth.

The usual statistical treatment of growth data of this type is to make an analysis of variance in weight at a given age and of final weight or net gain in weight of the experimental lots, and to make T tests between lots. The level at which T values cease to be significant is concluded to represent the minimum requirement. This is not a valid statistical method because it involves segmentation of the data, and the differences, which with finite numbers are insignificant, become significant when infinite numbers of experimental animals are used. In the present paper, maximum and minimum requirements for growth are considered synonymous and the level of vitamin A reported as inducing the greatest growth response may be considered the minimum or maximum amount required by growing turkeys.

With this in mind it is possible to calculate the point of greatest response to vitamin A, which, as is shown in figure 1, is at the level of 1,760, 1,512, and 1,776 I.U. of vitamin A per 100 gm of feed for the three experiments, respectively. The average of these is 1,683 I.U. of vitamin A per 100 gm of feed.

From these data it is concluded that 1,700 I.U. of vitamin A per 100 gm of feed represents the optimum level of this

vitamin for growing turkeys. This corresponds well with the results of Hinshaw and Lloyd ('34), who reported 1,736 I.U. of vitamin A per 100 gm of feed as meeting the minimum requirement of growing turkeys, and is far above the recommendations of other investigators.

No conclusive results were obtained from the histological examination of the beaks.

As is shown in figure 2, the concentration of vitamin A in the blood plasma of birds 6 weeks of age increased as the level of vitamin A in the diet was increased. This increase was quite rapid between lots 2 and 4, reaching more or less a plateau in lot 5, which received 1,200 I.U. of vitamin A per 100 gm of feed. A comparison of liver storage among birds in the 8 experimental lots is shown in figure 2. Liver storage of vitamin A increased rapidly between lots 2 and 5, and at a diminishing rate thereafter.

Figure 3 shows the average concentration of vitamin A per gram of liver, by lots, for birds two, 4, 6 and 8 weeks of age. Lots receiving less than 1,200 I.U. of the vitamin per 100 gm of feed showed a depletion of liver stores between the second and 4th week, with liver concentration of vitamin A remaining at a fairly constant level thereafter. Lot 5, except for a slight drop at 4 weeks, maintained its liver vitamin A at about the two-week level. Increased storage of vitamin A with increased age was found only in lots 6, 7 and 8, the amount of storage increasing as dosage was increased.

These data are very much in accord with the results of the analysis of the growth data. Plasma vitamin A concentration reaches a plateau when 1,200 I.U. of the vitamin per 100 gm of diet are fed, the amount given lot 5, and liver stores of vitamin A are maintained in a steady state at this level. Lots receiving less than 1,200 I.U. of vitamin A per 100 gm of feed responded to increases of vitamin A in the diet with large increases in the blood plasma level of the vitamin. These lots were also unable to maintain their stores of vitamin A in the liver. Additional increases of vitamin A in the diet, above 1,200 I.U., resulted in insignificant increases in the plasma level of vita-



min A but enabled the birds to store the vitamin in the liver at a rate proportional to the increased dose.

This suggests a minimum physiological requirement of turkey poults, as applied to liver storage and plasma levels, of 1,200 I.U. of vitamin A per 100 gm of feed, with an indicated optimum between 1,200 and 2,400 I.U., which optimum correlates well with the 1,700 I.U. of the vitamin which the growth data reveal as the level inducing the greatest response.

#### SUMMARY

1. Three experiments were conducted in which 228 male Broad Breasted Bronze turkeys were used to determine the vitamin A requirement of growing turkeys, the experiments being so designed as to allow the application of three criteria: growth, histopathology, and vitamin A concentration in the blood and liver.

2. A basal vitamin A deficient diet was used which, when adequately supplemented with vitamin A, was capable of producing birds averaging 2,000 gm in weight at 8 weeks of age. This diet was supplemented with levels of 0, 150, 300, 600, 1,200, 2,400, 4,800 and 7,200 I.U. of vitamin A per 100 gm of feed.

3. From the analysis of growth curves, the point of greatest response was calculated to be 1,683 I.U. of vitamin A per 100 gm of feed.

4. Histological examination of the brain revealed no conclusive results.

5. The plasma level of vitamin A in lots receiving less than 1,200 I.U. of the vitamin per 100 gm of feed was greatly influenced by the dosage of vitamin A in the diet. Lots receiving more than 1,200 I.U. of vitamin A per 100 gm of feed showed little effect of the dosage on plasma levels.

6. Lots receiving less than 1,200 I.U. of vitamin A per 100 gm of feed were unable to maintain their liver stores of the vitamin with increased age, while lots receiving more than 1,200 I.U. per 100 gm of feed increased their liver stores as age advanced.

7. It is concluded on the basis of the growth data and the plasma levels and liver storage of vitamin A that the vitamin A requirement of growing turkeys is 1,700 I.U. of the vitamin per 100 gm of feed.

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# NITROGEN BALANCE IN MEN CONSUMING RAW OR HEATED EGG WHITE AS A SUPPLE- MENTAL SOURCE OF DIETARY PROTEIN<sup>1</sup>

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Reports by Schwimmer and associates ('47, '48) indicate that for men consuming diets low in calories (approximately 1,800 Cal. daily) and drinking small quantities of water (800 ml daily), dried egg white provides an excellent source of available nitrogen. Because of this, it was decided to incorporate dried egg white as a major source of protein into certain experimental food items for trial by the Armed Forces. However, it was questionable whether the moderate degree of heat that could be applied in preparation of these items would be sufficient either in degree or duration to destroy the anti-trypsin which is present in commercial egg white powder. This possibility seemed to be a real one in view of Harte's observations ('45). He found by *in vitro* investigations that commercially dried egg white contained as much anti-tryptic principle as undried egg white, and that the digestibility of these products was inferior to that of coagulated egg white as determined by *in vitro* measurement.

In the present communication we report observations made to determine the importance of egg white anti-trypsin in human

<sup>1</sup> The opinions expressed in this paper are those of the authors, and do not necessarily represent the official views of any governmental agency.

nutrition. Nitrogen balance was measured in men who consumed the following proteins as supplemental sources of dietary nitrogen: (a) lactalbumin; (b) unheated commercial dried egg white; (c) heated commercial dried egg white; and (d) heated commercial dried egg white plus its natively equivalent amount of chemically isolated anti-trypsin (ovomucoid). The last named substance was studied in an attempt to determine specifically the relationship between the naturally occurring anti-trypsin and the chemically isolated form.

## METHODS

### *General*

Two healthy, young, volunteer, male subjects were studied for approximately 6 weeks in a metabolic ward in a research hospital. The subjects were allowed freedom of activity in the ward. One subject worked in a hospital laboratory during the day but was not physically very active. The other was allowed to leave the hospital three times a week to visit friends but exercise was kept at a minimum. Temperature, pulse, respiration, body weight, and basal blood pressure were recorded each day, as was the general state of health. Special care was taken to note any symptoms or reactions that might be related to consumption of the diets. All urine and stools (with carmine markers) were collected and stored under refrigeration. The 24-hour urine output was recorded, three-day composite specimens were prepared with toluene (2 ml per 100 ml) as the preservative, and aliquots were taken for analysis of total nitrogen. The stools were mixed in a Waring Blendor with distilled water and aliquots taken for total nitrogen determination.

### *Diet and fluid intake*

Each subject subsisted on a fixed basal diet and a constant fluid intake (2,500 ml/day). The basal diet consisted of 4 well varied menus of natural foods; each menu was rotated in the same sequence during each of the 5 experimental periods described below. An example of one of the menus follows:

*Breakfast:* orange juice; applesauce; puffed rice and cream; bread, butter and cinnamon sugar; beverage. *Dinner:* apple juice; Spanish rice; broccoli, frozen; fresh vegetable salad with dressing; olives; crackers, butter and jelly; orange ice; and beverage. *Supper:* pineapple juice; vegetable salad; corn, canned; beets, canned; green beans, canned; tomato, fresh; lettuce and pickle, with dressing; crackers, butter and jelly; Jello and banana; and beverage. All food items were carefully weighed and prepared by the dietitian. There was no plate waste.

The basal diet was supplemented with 250  $\mu$ g of biotin and one multiple vitamin capsule daily. Each capsule provided: thiamine, 2.0 mg; riboflavin, 2.0 mg; niacinamide, 10.0 mg; pyridoxine, 0.2 mg; calcium pantothenate, 3.0 mg; choline, 20.0 mg; inositol, 10.0 mg; folic acid, 10  $\mu$ g. The basal diet was calculated to contain the following as an average: Calories, 2,447; protein, 26.9 gm; fat 88.6 gm; carbohydrate, 385.6 gm; iron, 11.5 mg; vitamin A, 13,076 I.U.; thiamine, 3.0 mg; riboflavin, 3.7 mg; niacin, 19.0 mg; vitamin C, 265 mg. Duplicate meals from each of the 4 menus were analyzed for nitrogen content; this was done in each period described below. The test proteins were added to drinks and cooked dishes in such proportions as to be almost undetectable. The amounts used were as follows:

*Period I (9 days):* Unheated lactalbumin, 30 gm (66.25% protein).

*Period II (9 days):* Unheated commercial egg white power, 25.8 gm (77.5% protein).

*Period III (9 days):* Heated commercial egg white powder, 25.8 gm (77.5% protein).

*Period IV (9 days):* Heated commercial egg white powder, 25.8 gm (77.5% protein) plus ovomucoid  $\frac{1}{4}$  gm.

*Period V (6 days):* Unheated lactalbumin 30 gm (66.25% protein).

The total protein intake was therefore approximately 50 gm per day. It will be noted that the periods when anti-trypsin was present were alternated with periods when it was absent.

This arrangement tended to eliminate any "metabolic lag" that might arise due to the former period.

Some results of studies on the *in vitro* digestibility of the test proteins are shown in table 1.

It is apparent that the *in vitro* anti-tryptic activity of the dried egg white was high, and that wet autoclaving was necessary to cause improvement in digestibility to that of lactalbumin. This method was therefore used in preparing the heated egg white for periods III and IV. The ovomucoid used in period IV was prepared according to the method described by Lineweaver and Murray ('47). That this substance also had high *in vitro* anti-tryptic activity is shown by data presented in table 2.

TABLE 1

*Studies on the in vitro digestion<sup>1</sup> of egg white and lactalbumin by trypsin*

DAY OF DIGESTION	UNHEATED COMMERCIAL EGG WHITE POWDER % Digestion	COMMERCIAL EGG WHITE POWDER, AUTOCLAVED DRY % Digestion	COMMERCIAL EGG WHITE POWDER, AUTOCLAVED WET % Digestion	POWDERED LACTALBUMIN, UNHEATED % Digestion
1	2.8	3.7	30.0	42.8
4	5.5	6.2	26.6	29.0
5	5.0	7.8	24.1	25.6
6	6.1	7.1	23.8	25.1
7	6.2	6.5	22.7	22.9

<sup>1</sup> Per cent digestion =  $\frac{\text{increase in titratable amino N} \times 100}{\sqrt{10.15 \text{ days} \times \text{gm} \times 0.01 \times \% \text{ protein}}}$ . See Freed et al. ('49).

### *Clinical and biochemical aspects*

Clinical and biochemical laboratory observations were made at regular intervals as follows: (a) *Blood serum* with respect to total protein (Ma and Zuazaga, '42), albumin (Howe, '21a, b), non-protein nitrogen (Daly, '33), cholesterol esters (Sperry and Brand, '43); (b) *blood*, for hematocrit, hemoglobin, red and white cell counts (Todd and Sanford, '43); and (c) *urine* with respect to albumin, which was determined qualitatively on random samples.

The determinations of total nitrogen in the food specimens, three-day feces, and three-day urine collections were per-

formed by the micro-Kjeldahl method of Ma and Zuazaga ('42).

### RESULTS AND CONCLUSIONS

There were no significant changes in body weight, hemoglobin, serum proteins, blood count, cholesterol, non-protein nitrogen, or systolic or diastolic blood pressure.

On the basis of the results presented in table 3, certain conclusions may be drawn concerning nitrogen absorption and nitrogen balance:

TABLE 2

*Effect of chemically prepared anti-trypsin (ovomucoid) upon in vitro digestion of autoclaved commercial egg white powder*

DAY OF DIGESTION	HEATED EGG WHITE	HEATED EGG WHITE PLUS ANTI-TRYPSIN
	<i>Titration units</i>	
1	2.58 (19.8%) <sup>1</sup>	— 0.40
2	4.02	— 0.06
4	4.55 (17.5%) <sup>1</sup>	— 0.13
7	5.15 (15.0%) <sup>1</sup>	0.55

<sup>1</sup> Values in parentheses refer to per cent digestion of substrate per day calculated by the Schultz rule, as in table 1.

(a) As measured by three-day fecal nitrogen, unheated commercial egg white powder was absorbed to a somewhat lesser extent than the cooked form, or the cooked form plus ovomucoid; however, its absorption was not markedly less than that for lactalbumin.

(b) A more negative nitrogen balance occurred with unheated commercial egg white powder than when the heated form was used, or when ovomucoid was added to heated egg white; however, the nitrogen balance from the unheated egg white powder was in turn less negative than that obtained when a corresponding amount of lactalbumin was used.

(c) When ovomucoid was isolated from other proteins of egg white and then fed in proportionate amounts with heated dried egg white, there were only negligible (if any) adverse effects upon absorption of the egg white and upon nitrogen

TABLE 3

*Nitrogen balance (average by three-day sub-periods) for two subjects during feeding with supplements of lactalbumin, unheated egg white, heated egg white, and heated egg white plus ovomucoid*

	PERIOD <sup>1</sup>				
	I	II	III	IV	V
<b>A. Supplement</b>					
Type of protein	Lactal- bumin un- heated	Egg white un- heated	Egg white heated	Egg white heated (a) ovo- mucoid (b)	Lactal- bumin un- heated
% of total N intake accounted for by N of supplement	41.2	40.8	41.2	38.0 (a) 7.7 (b)	41.2
<b>B. Diet</b>					
1. Basal diet, gm N	13.62	13.80	13.56	13.62	13.62
2. Test proteins, gm N	9.54	9.51	9.51	9.51	9.54
3. Ovomucoid, gm N	0	0	0	1.92	0
4. Total diet, gm N	23.16	23.31	23.07	25.05	23.16
<b>C. Excretion</b>					
1. Feces, gm N					
(a) Subject 1	6.30	7.39	5.92	5.97	7.40
(b) Subject 2	4.83	5.31	3.27	4.73	4.23
2. Urine, gm N					
(a) Subject 1	23.3	19.3	18.3	19.5	22.8
(b) Subject 2	21.2	20.1	19.2	20.5	18.8
<b>D. Absorption</b>					
1. Absorption, gm N					
(a) Subject 1	16.86	15.92	17.15	19.08	16.84
(b) Subject 2	18.33	18.0	19.8	20.32	18.93
2. Absorption, % of intake					
(a) Subject 1	72.8	68.3	74.3	76.2	73.1
(b) Subject 2	79.1	77.2	85.8	81.1	81.7
<b>E. Retention</b>					
1. Nitrogen retained, gm N					
(a) Subject 1	- 6.44	- 3.38	- 1.15	- 0.42	- 7.04
(b) Subject 2	- 2.87	- 2.10	+ 0.60	+ 0.18	+ 0.13

<sup>1</sup> Each period lasted 9 days except period V, which lasted 6 days.



retention, as measured by nitrogen excretion in feces and urine.

(d) Therefore, it follows that there can be no direct application to human feeding programs of results obtained from *in vitro* studies of the digestibility of unheated commercial egg white powder. It is concluded that egg white anti-trypsin exerts very little deleterious effect upon nitrogen balance in man under the conditions herein described.

(e) As compared with lactalbumin, heated dried commercial egg white powder was a protein of high biologic value when used as a supplement to the basal diet.

#### DISCUSSION

Vernon ('04) first reported that fresh egg white contained an anti-tryptic substance. Thereafter, Falta ('06), Hamalainen and Helme ('07), Cathcart and Green ('13), Mendel and Lewis ('13), Bateman ('16) and others described many of the effects found upon feeding raw egg white to animals. The anti-tryptic activity of egg white has been attributed variously to: (a) the resistance of the egg white proteins to digestion, and preferential absorption of egg white on the enzyme; (b) the egg white globulins; and (c) a hydrolysis product of egg white proteins. Concerning the chemical nature of egg white anti-trypsin little was known until Balls and Swensen ('34) reported that it contains nitrogen and has an optical rotation similar to that of ovomucoid. Meyer and co-workers ('36) indicated that a highly active anti-trypsin preparation of Swensen's had the properties and composition of an egg mucoid. It remained for Line-weaver and Murray ('47) to show that the anti-tryptic activity of egg white appeared quantitatively in its mucoid fraction, that ovomucoid could not be fractionated into components of high and low anti-tryptic activity, that electrophoresis failed to reveal the presence of another protein in ovomucoid, that less than one molecule of ovomucoid was necessary to produce 50% inhibition of one molecule of trypsin, and that heat-denatured ovomucoid had little or no anti-tryptic activity. It

was thus concluded that egg white anti-trypsin was identical with ovomucoid.

Ovomucoid is a relatively stable protein. While anti-tryptic activity is destroyed by heat, this denaturation takes place at a rate much slower than that for most proteins. Lineweaver and Murray ('47) point out that in an aqueous solution, at pH between 3.0 and 7.0, heating at 80°C. for 30 minutes produces no essential destruction of anti-tryptic activity, but at pH 9.0 at the same temperature all the activity is lost. Boiling an egg for three minutes results in a loss of 35% of the activity, while 75% is lost in an egg boiled for 10 minutes. In the dry form, inactivation takes place more slowly, but heating for 18 hours at 60°, 110°, and 140°C. causes activity losses of 40, 94, and 100%, respectively.

It is well known that the application of heat to many proteins changes "digestibility." In most instances this is due to a partial digestive effect of the heat *per se*. However, where an anti-tryptic factor is present, such as occurs in egg white or in soybeans and other legumes, this too is destroyed by heat. The proportionate effects of heating *per se* and the removal of anti-trypsin are not known. Consequently, human feeding studies of raw versus heated egg white of the type reported by Rose and McLeod ('22), or of raw versus autoclaved soybean as reported by Lewis and Taylor ('47), while providing valuable data on the over-all effect of heat on these raw foods, do not clearly delineate the significance of their respective anti-tryptic substances. It was for this reason that the design of the present experiment included one period when a proportionate amount of chemically isolated ovomucoid was added. It was necessary, of course, to determine that no change had taken place in the anti-tryptic character of the ovomucoid, and this was done by *in vitro* testing as is shown in table 2.

From the results of the feeding tests it appears that although unheated commercial egg white may have considerable anti-tryptic activity *in vitro*, yet it does not seem to have any markedly deleterious effect upon nitrogen retention by adults.

These findings are similar to those reported by Lewis and Taylor ('47), who fed raw and autoclaved soybean as the sole source of dietary protein. For these reasons it is apparent that the results of *in vitro* testing of the digestibility of foods containing anti-tryptic substances cannot be applied to human feeding programs without additional testing in man. In the case of unheated commercial egg white powder we have an example of a protein which on the basis of *in vitro* testing would be expected to have a low biologic utilization value, yet actually it was found to be somewhat superior in this respect to one of the widely accepted standard proteins (lactalbumin) when tested by nitrogen balance measurements.

#### SUMMARY

1. A study was conducted of the effects of ingested anti-tryptic factor upon nitrogen balance in man.

2. Two subjects living in a well-standardized hospital environment consumed daily a standard diet containing 2,500 Cal. and approximately 50 gm of protein. About 40% of the protein intake was comprised of a test protein during each of 5 periods, as follows: Period 1, lactalbumin (9 days); period 2, unheated commercial egg white powder (9 days); period 3, heated commercial egg white powder; period 4, as in period 3, plus a small quantity of chemically isolated ovomucoid, the egg white anti-tryptic factor (9 days); period 5, lactalbumin (6 days).

3. There were only minor differences in the amounts of nitrogen absorbed during these periods. Nitrogen retention was least favorable in the lactalbumin periods, and it appeared that chemically prepared ovomucoid had no unfavorable effect upon nitrogen utilization. Unheated commercial egg white, while less well utilized than the heated form, was nevertheless better than lactalbumin in this respect. It was concluded that egg white anti-trypsin exerts very little deleterious effect upon nitrogen balance in man under the conditions of the present experiment.

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# CALCIUM AND PHOSPHORUS REQUIREMENTS OF BREEDING BOBWHITE QUAIL

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The percentage of dietary calcium necessary for optimum reproduction in gallinaceous birds depends on the number of eggs laid, the quantity of feed consumed, and the phosphorus content of the diet (Branion, '38; Titus, '39; Ewing, '41). Egg-shell formation, compared with bone calcification, is an extremely rapid process. In the case of poultry, it is known that the calcium carbonate is secreted by the glands in the wall of the oviduct, a process requiring only 12 to 16 hours (Cruikshank, '35). In the absence of a sufficient supply of calcium, egg production is lowered, with the eggshells becoming progressively thinner. The serum calcium of the hen falls and there is a depletion of the calcium and phosphorus contents of the bones. Nevertheless, according to Branion, the percentage composition of the shell remains constant, showing that, as in bone formation, if the mineral is deposited, it is laid down in the normal proportion. Deobald et al. ('36) concluded that about 10% of the calcium stored in the bones may be called upon for egg production.

Branion points out that not as much research has been done with breeding birds as with chicks. Norris and associates ('33, '34) concluded that 1.65% of calcium is sufficient to meet the requirements of laying hens as judged by the egg production, shell strength, and ash, and by the levels of calcium and phosphorus in the blood. When the calcium level of the diet was

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1.8%, a level of 0.5% phosphorus was insufficient but 0.75% was adequate. Miller and Bearse ('34) were convinced that egg production is definitely affected by the quantity of phosphorus in the diet. They obtained their highest production with 0.80%, and found that 2.25% of calcium was adequate. According to Titus et al. ('37), pullets and hens differ in calcium requirements, and a high level of calcium, such as 5.4%, adversely affects hatchability. In disagreement with Miller and Bearse, these workers concluded that the level of phosphorus intake had no effect on egg production.

There is no report in the literature of work conducted on the calcium and phosphorus requirements of breeding gamebirds. Inasmuch as the bobwhite quail (*Colinus virginianus*) is one of the most popular gallinaceous upland gamebirds in the Eastern United States, an investigation was started with this species at the Patuxent Research Refuge, Laurel, Maryland, to determine its requirements for optimum reproduction. In these studies the effects of varying dietary levels of the two minerals, and of variations in the ratios between the two, have been determined.

#### EXPERIMENTAL

During the breeding seasons of 1946, 1947 and 1948, paired adult quail housed in individual breeding compartments were divided into groups of from 12 to 16 pairs each. Each group received a diet designed to meet the known requirements of breeding quail for protein, fat, fiber, total energy, and the various fat- and water-soluble vitamins. The diets differed in the percentages of phosphorus present, and in the Ca/P ratios. In the first series of tests (1946), each of the 6 diets studied contained 0.75% phosphorus, while the Ca/P ratios were 1:1, 1.69:1, 2.31:1, 3.1:1, 3.79:1, and 4.48:1. The same 6 Ca/P ratios were used in the second series (1947), but the phosphorus level of all diets in this series was 1.00%. In the third series (1948), the Ca/P ratio in each of the three diets was 2.33:1, with phosphorus levels of 0.75, 1.0, and 1.25%, respectively. The composition of the diets is presented in table 1.



TABLE 1

## Composition of diets

INGREDIENT	DILT NO.														
	46-1	46-2	46-3	46-4	46-5	46-6	47-1	47-2	47-3	47-4	47-5	47-6	48-1	48-2	48-3
	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%
Ground yellow corn	50.20	48.83	47.34	45.82	44.15	42.85	47.20	45.41	43.21	41.05	38.86	36.65	41.77	43.16	41.52
Standard wheat middlings	10.00	10.00	10.00	10.00	10.00	10.00	10.00	10.00	10.00	10.00	10.00	10.00	10.00	10.00	10.00
Soybean oil meal	22.00	22.21	22.54	22.86	23.38	23.51	24.00	24.50	25.00	25.50	26.00	26.50	25.00	25.00	25.00
Dried whey	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00
Liver meal	11.00	11.00	11.00	11.00	11.00	11.00	11.00	11.00	11.00	11.00	11.00	11.00	11.00	11.00	11.00
Salt mix <sup>1</sup>	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Vit. A and D oil (3,000 A — 425 D)	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Steamed bone meal	1.80	1.80	1.80	1.83	1.82	1.83									
Limestone		1.16	2.32	3.49	4.65	5.81		1.25	2.94	4.62	6.30	8.00	2.55	2.45	2.75
Tricalcium phosphate							2.80	2.81	2.82	2.83	2.84	2.85	1.68	3.39	4.73
Total	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
Total Ca <sup>2</sup>	0.75	1.27	1.80	2.32	2.85	3.37	1.17	1.67	2.33	3.00	3.66	4.33	1.76	2.33	2.92
Total P <sup>2</sup>	0.75	0.75	0.75	0.75	0.75	0.75	1.00	1.00	1.00	1.00	1.00	1.00	0.75	1.00	1.25
Ca/P	1.00	1.69	2.31	3.09	3.79	4.48	1.17	1.67	2.33	3.00	3.66	4.33	2.33	2.33	2.35

<sup>1</sup>The salt mixture consisted of iodized common salt (NaCl) 100 gm, and anhydrous manganese sulfate (MnSO<sub>4</sub>) 1.7 gm.

<sup>2</sup>Based upon analysis of finished diets.

Records were kept of the body weights of the birds, food consumption, egg production, fertility and hatchability. Eggs were collected three times per week and stored in a cooling shed before being incubated. No eggs were kept over two weeks before being incubated.

During the periods June 9 to 14 and July 13 to 19, 1946, all eggs were saved for chemical analysis to determine the effects of dietary variations on the calcium and phosphorus contents of the shells, shell membranes, and yolks and whites.

The results of these studies are shown in tables 2 and 3. Inasmuch as there may have been some slight differences in the genetic backgrounds of the birds, or in the management technics employed in different years, it appears inadvisable to make direct comparisons between results obtained in different seasons.

### *Mortality*

The highest mortality rate (4.2%) occurred on the diets having the highest calcium content. However, there did not appear to be any dietary effect and the mortality rate did not appear abnormal.

### *Live weights*

The body weights of the birds were apparently unaffected by the Ca/P ratios, or by the levels of calcium and phosphorus in the diets. The observed changes in weight were normal for breeders.

### *Feed consumption*

The consumption of food increased directly with the increase of calcium in the diet. Exceptions to this statement may be noted in the cases of diets 48-2 and 48-3.

### *Egg production*

In each of the first two experiments the highest egg production was obtained on diets with a Ca/P ratio of 2.33:1. When the phosphorus content of the diet was 0.75%, the differences in production between the 2.33:1 ratio and the ratios of 1:1,

TABLE 2  
*Results of feeding breeding quail diets varying in calcium and phosphorus content*

RESULTS	DILF NO.														
	46-1	10-2	46-3	46-1	10-5	10-6	17-1	47-2	47-3	17-4	47-5	47-6	18-1	48-2	18-3
Mortality (%)	1.4	1.1	2.8	1.1	0	1.4	0	2.8	2.8	2.8	4.2	4.2	0	0	0
Av. weight change (gm)	-7	-6	-7	-11	-9	-9	+2	+3	+1	-5	-2	-2	+3	+1	+5
Av. food consumption (gm)	15.8	16.3	16.4	16.4	16.7	16.7	16.4	16.6	17.6	17.5	17.4	18.0	16.9	18.5	18.1
Av. no. eggs/hen	31	41	56	47	49	37	56	56	72	51	45	53	67	61	56
Defective egg-shells (%)	27	16	7	11	12	7	16	9	10	12	6	7	2	1.4	1.2
Fertility (%)	93	87	89	86	81	92	89	83	83	85	86	79	87	92	89
Hatchability (%)	66	68	69	69	67	64	72	50	60	62	58	62	68	55	61

1.69:1, 3.79:1, and 4.48:1 are all significant statistically (odds of 99 to 1 in the case of the 1:1 ratio). However, the differences resulting when the phosphorus content of the diet was 1.00% were not significant. When the 1946 results are compared with those obtained in 1947, it appears that egg production on diets containing 1.00% phosphorus was significantly higher than that on diets containing 0.75%. However, no significant differences resulted when the two levels were tested simultaneously in 1948.

TABLE 3

*Calcium and phosphorus content of eggs produced by quail fed diets of 6 different Ca/P ratios at a phosphorus level of 0.78%*

COMPONENT	COLLECTION DATE	CA/P RATIO					
		1:1	1.69:1	2.3:1	3:1	3.7:1	4.3:1
Shells and shell membranes							
Calcium (%)	June 9-14	30.1	30.3	31.0	31.1	31.9	31.7
	July 13-19	18.0	26.4	23.6	30.0	30.3	33.8
Phosphorus (%)	June 9-14	0.20	0.21	0.20	0.20	0.20	0.18
	July 13-19	0.60	0.44	0.42	0.23	0.13	0.14
Yolks and whites							
Calcium (%)	June 9-14	0.22	0.23	0.21	0.31	0.30	0.30
	July 13-19	0.34	0.25	0.28	0.23	0.33	0.24
Phosphorus (%)	June 9-14	0.31	0.30	0.27	0.31	0.29	0.29
	July 13-19	0.73	0.77	0.69	0.51	0.79	0.83

### *Eggshell condition*

In each of the first two years the percentage of defective shells (cracked, checked, rough or abnormal) was greatest on diets containing a Ca/P ratio of 1:1. The differences were less marked when the diets contained 1.00% of phosphorus.

### *Egg fertility*

Fertility was unaffected by the Ca/P ratio, or by the levels of calcium and phosphorus in the diets.

*Egg hatchability*

The highest hatchability occurred on the ratios of 2.3:1 and 3:1, when the phosphorus level of the diet was 0.75%. On diets containing 1.00% phosphorus the highest hatchability occurred on the Ca/P ratio of 1:1. However, there were no significant differences in any of the experiments.

*Calcium and phosphorus content of eggs*

The results of chemical analyses on eggs collected during the weeks of June 9 to 14 and July 13 to 19, 1946, are presented in table 3. The average calcium content of the yolk and white of all eggs, regardless of the diet, was 0.27%, or 0.02 gm per egg, and that of the shell and shell membrane was 29.17%, or 0.94 gm, making the calcium content of an entire egg weighing approximately 9.0 gm, 10.7%, or about 1.00 gm.

Neither the Ca/P ratio of the diet nor the period of the season during which the eggs were laid had any significant effect on the calcium content of the yolks and whites. During the early part of the laying season the Ca/P ratio of the diet had only a slight effect on the percentage of calcium in the shell and shell membrane, but a significant effect was noted later in the season. The calcium content of the shells from birds on the 1:1 ratio dropped 40.0%, and that of the shells from birds on the 4.33:1 ratio increased 6.0%.

The average phosphorus content of the yolk and white of all the eggs was 0.50% and that of the shell and shell membranes, 0.26%, or 0.008 gm, making the total phosphorus content of the egg approximately 0.05 gm.

During the week of June 9 the phosphorus content of the eggs was unaffected by the variations in the calcium content of the diet. However, during the week of July 13 the phosphorus content of the yolks and whites was double that of the previous period, but apparently was unaffected by the level of dietary calcium. There was a reverse correlation between the phosphorus content of shells and shell membranes collected during the later period and the level of dietary calcium. When the

Ca/P ratio in the diet was either 1:1, 1.67:1, or 2.3:1, the phosphorus content of the shells and shell membranes was significantly higher in July than in June, whereas when the ratio was greater than 3:1 the reverse situation was noted.

#### DISCUSSION

As a result of their studies with chickens, Titus and associates ('37) developed an equation for estimating the percentage of calcium required in the diet for any given rate of egg production. This equation, modified for bobwhite quail, is expressed as follows:

$$Ca = \frac{1.292 (PF - 5.0 E) + 100 E}{F},$$

in which Ca = the required percentage of calcium in the diet; 1.292 = the ratio of calcium to phosphate in di-calcium phosphate, the form in which phosphorus appears in the diet; P = the percentage of phosphorus in the diet; F = the number of grams of feed consumed by a laying quail during the year (approximately 5475 gm); 5.0 = 100 times the quantity of phosphorus (0.05 gm) in the average egg; E = the number of eggs produced in one year (for quail in captivity, an average of 60 eggs); and 100 = 100 times the quantity of calcium (1.00 gm) in an average egg.

With the phosphorus level at 0.75%, the optimum level of calcium in the diet should be 2.0% according to this equation. The actual levels of calcium tested which were nearest this quantity were 1.8 and 2.3%. The highest egg production occurred on the 1.8% level (Ca/P ratio of 2.33:1), but the difference between it and production on the 2.3% level was not statistically significant. Similarly, the highest hatchability of eggs occurred on these two levels.

With the phosphorus level at 1.00%, the optimum level of calcium in the diet should be 2.32% according to the equation. In the second experiment the highest egg production occurred on the calcium level of 2.33%. Therefore, in the first two experiments at least, the actual results tend to agree with the

calculated value for the optimum level of dietary calcium when the phosphorus is maintained at either 0.75 or 1.00%.

Branion ('38; see also Ewing, '41) was skeptical of the worth of the equation on the ground that it does not make full allowance for variations in the rate of production or size of egg. Inasmuch as any such formula is based upon the flock average, its use for incorporating a fixed quantity of mineral into a diet will cause high-producing hens to be underfed and low producers, overfed. While Branion's objection probably is valid for chickens whose range in production and egg size may be great, it should not be very important in the case of quail. The latter lay eggs that vary little in weight and seldom number more than 100 per breeding season for birds in captivity.

From these data it would appear that the calcium and phosphorus requirements of breeding quail may be supplied by diets containing 0.75% phosphorus and 1.8% calcium (Ca/P ratio of 2.33:1). No significant change in the condition of the breeders, egg production, fertility, hatchability, or survival of offspring during the first 5 days after hatching resulted when the phosphorus level in the diet was increased. The ratio of calcium to phosphorus appears quite important in diets containing 0.75% phosphorus, but the effects of changes in the Ca/P ratio are less marked when the experimental diets contain 1.00% phosphorus.

However, observations made in the course of other studies during the winter of 1948-49 indicate that the criteria used in the evaluation of results may have been inadequate. All chicks hatched during the 1948 season were reared on a normal diet, and were divided into groups of 20 birds each on November 1. Distribution into groups was on the basis of the summer diets of the parents of the experimental birds, in that the parents of approximately one-third of the birds in each group had been fed diet 48-1 (0.75% phosphorus); the parents of an equal number had received diet 48-2; while the parents of the remaining birds had received diet 48-3. The diets fed during the winter supplied adequate amounts of protein, fat, carbohydrate

and minerals, but varied in their contents of vitamin A, vitamin K, and the various members of the vitamin B complex. The only known differences among the experimental birds were the dietary backgrounds of the parents. It was found that 23.6% of the chicks from parents fed diet 48-1 (0.75% phosphorus, 1.76% calcium) died during the 20-week experimental period, and that the mortality rates from diets 48-2 and 48-3 were 13.3% and 8.5%, respectively. On autopsy, blood clots were found in the viscera or brain of 27% of the birds from parents fed diet 48-1, in 17% of the birds from diet 48-2, and in 15% of the birds from diet 48-3.

Presumably, these differences in the winter mortality rates of the young birds may have resulted from the differences in the diets fed the parents during the breeding season. If this is the case, it may be concluded that while a diet containing 0.75% phosphorus and 1.8% calcium is adequate to permit reproduction at a normal rate, it is inadequate to insure survival of the offspring during the winter months. As judged by the criteria of the survival and weight maintenance of the parent birds, by egg production, fertility, hatchability and survival of the offspring, it appears that breeding bobwhite quail require diets furnishing approximately 1.00% phosphorus and 2.3% calcium.

#### SUMMARY

In the course of studies designed to determine the calcium and phosphorus requirements of breeding bobwhite quail, it was found that best results were obtained when the Ca/P ratio in the diet was approximately 2.3:1. Variations in the Ca/P ratio produced significant differences in results when the level of phosphorus in the diet was 0.75%, but the differences were less marked when the level of phosphorus was increased to 1.00%.

Although diets containing 0.75% phosphorus and 1.8% calcium appeared adequate for reproduction, as judged by the criteria of the maintenance of satisfactory condition in the breeders, egg production, fertility, hatchability and survival



of offspring during the first 5 days after hatching, it was found that the winter mortality of the offspring of birds fed such a diet was much greater than that occurring in the offspring of birds fed on diets containing 1.00 or 1.25% phosphorus. It is concluded that breeding bobwhite quail require diets furnishing approximately 1.00% phosphorus and 2.3% calcium.

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# SUCCINYLSULFATHIAZOLE AND A RAT GROWTH FACTOR IN LIVER <sup>1</sup>

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In connection with another investigation it was noted that rats on a purified diet containing synthetic pteroylglutamic acid (PGA) and 5 gm of succinylsulfathiazole (sulfasuxidine) per 100 gm of diet did not grow <sup>2</sup> so well as rats on the same diet but with the sulfasuxidine and PGA omitted. This difference in growth (weight gain) was not large, but it was consistent. These few observations led to a more detailed study of the factors concerned with this difference. The results of this study are given below.

## EXPERIMENTAL

Albino rats, raised in the laboratory, were used as experimental animals. For the most part, the animals were started on the experimental diets at about 25 days of age, and the duration of the experiments was 6 weeks. Only those litters which had as many animals of one sex as there were groups in an experiment were used. Each group was composed of one animal from each of 6 or 7 litters and all animals of any

<sup>1</sup> Most of the data presented here were collected during 1946 and the first three months of 1947, but the completion of the experimental work and preparation of the manuscript were unavoidably delayed until recently.

<sup>2</sup> Measured as individual weights only.

one litter were of the same sex.<sup>3</sup> The animals were kept in individual cages and weighed daily. The diets were prepared in small amounts and stored in closed containers in the cold room until used or discarded. In the first two of the three experiments reported here, total white cell and differential counts and hemoglobin determinations were made during the first three weeks of the experiments and again during the second three weeks. These determinations were usually made toward the end of each of the two periods. Only the counts taken during the second three weeks are reported here.

### *Experiment 1*

There were 6 groups of animals in the first experiment. The diets used are given in table 1. The casein of these diets was extracted in the laboratory by cold percolation with about 6 changes of the extracting fluid per week. The casein was first extracted with 95% ethyl alcohol, then 80% alcohol, and finally 50%. In each case the extraction was continued until the effluent was nearly colorless.

The liver preparation<sup>4</sup> was the 70% alcohol soluble fraction of the material obtained from hog liver by hot water extrac-

<sup>3</sup> When this system of distribution is used, any two animals of the same litter can be considered a pair, and any two groups within a given experiment can be compared statistically by determining the standard error of the differences in weight gains between the two members of all pairs that make up the two groups.

Thus,

$$\sqrt{\frac{\sum (d^2)}{(N-1)N}} = \text{Standard error, and}$$

$\frac{\text{Mean of the differences}}{\text{Standard error}} = "t,"$  where  $d$  is the deviation of each difference from the mean of the differences and  $N$  is the total number of pairs.

This method of distribution of the animals, when growth is the criterion, and also this procedure for the calculation of the degree of significance, are not only highly sensitive but they decrease the error when males and females are used in the same experiment.

The difference between two means was considered significant if the probability that the results were due to chance was 0.05 or less.

<sup>4</sup> The authors wish to thank Parke, Davis and Co., Detroit, for the crude liver extract; Merck and Co., Inc., Rahway, N. J., for most of the B vitamins; Lederle Laboratories, Inc., Pearl River, New York, for the pteroylglutamic acid; and Sharp and Dohme, Inc., Glenolden, Pa., for the succinylsulfathiazole and antipernicious anemia liver concentrate.

tion which represented 50 gm of fresh liver per gram of extract.

The first group of animals of experiment 1 was given the basal or no. 1 diet, i.e., the PGA, sulfasuxidine and liver extract were all omitted. All other groups received this diet plus one or more supplements. The second group received sulfasuxidine, while groups 3, 4, and 5 were given sulfasuxidine and

TABLE 1  
*Diets used in experiment 1*

INGREDIENTS <sup>1</sup>	GROUP NO.					
	1	2	3	4	5	6
Casein (alcohol ext.) <sup>2</sup> (%)	18	18	18	18	18	18
Salt mixture <sup>3</sup> (%)	4	4	4	4	4	4
Cottonseed oil (%)	2	2	2	2	2	2
Cellulose <sup>4</sup> (%)	2	2	2	2	2	2
Liver extract <sup>5</sup> (%)						
Sucrose (%)	74	74	74	74	74	72
PGA <sup>6</sup> (mg %)			30	60	120	
Sulfasuxidine <sup>6</sup>		+	+	+	+	+

<sup>1</sup> In addition, each diet contained the following vitamins in mg %: Thiamine chloride, 0.5; riboflavin, 0.5; pyridoxine, 0.5; calcium pantothenate, 3.0; *D*-inositol, 3.0; nicotinic acid, 3.0; 2-methyl-1,4-naphthoquinone, 0.025; choline chloride, 200. Approximately 0.5 ml of cod liver oil was given by mouth to each animal twice weekly and 0.4  $\mu$ g of biotin by intraperitoneal injection three times per week.

<sup>2</sup> See text.

<sup>3</sup> Jones and Foster ('42).

<sup>4</sup> Cellu flour.

<sup>5</sup> Pteroylglutamic acid.

<sup>6</sup> Succinylsulfathiazole; 5 gm per 100 gm of diet.

varying amounts of PGA (table 1), and group 6 was given sulfasuxidine and 2% of the liver extract. It was thought advisable to give the PGA at more than one level in the event that the large amount of sulfasuxidine would cause a partial inactivation or incomplete absorption of this vitamin.

The biotin used in the first experiment was made up as a concentrated solution in alcohol at the beginning of the experiment. A small portion was diluted with water at approximately weekly intervals. To determine if the results in respect

to growth were due to a decrease in activity of the biotin stock solution, it was analyzed for the vitamin at the end of the experiment. There was no detectable loss.

*Results.* Weight gains and white cell counts are given as averages in table 2. Most of the animals of group 2 (sulfasuxi-

TABLE 2

*Initial weights, weight gains and white cell counts of experimental animals*

GROUP AND DIET NO.	AVERAGE INITIAL WEIGHT	AVERAGE WEIGHT GAIN	AVERAGE WHITE CELL COUNT
	gm	gm	
Experiment 1, 6 males, 1 female in each group			
1 <sup>1</sup>	49.1	150.1	18,200
2	50.6 <sup>2</sup>	56.9	8,800
3	51.4	125.6	18,900
4	50.8	115.9	19,200
5	50.8	119.9	18,300
6	50.2	153.1	16,700
Experiment 2, 2 males, 5 females in each group			
7 <sup>3</sup>	44.3	136.7	16,000
8	44.4 <sup>2</sup>	46.6	7,400
9	44.4	107.7	17,400
10	44.4	138.0	18,000
Experiment 3, 6 males in each group			
11 <sup>4</sup>	51.5	156.7	
12	51.2 <sup>2</sup>	48.2	
13	51.5	128.2	
14	50.5	120.3	
15	50.8	160.2	

<sup>1</sup> See table 1 for diets used in first experiment.

<sup>2</sup> As most of the animals in this group lost weight toward the end of the experiment, the maximum weight attained was used for calculating the weight gain.

<sup>3</sup> The diets used in experiment 2 were the same as diets 1, 2, 4 and 6 of experiment 1 with the following changes: The amounts of thiamine chloride, riboflavin, pyridoxine, calcium pantothenate, *i*-inositol, nicotinic acid, and 2-methyl-1,4-naphthoquinone were doubled and biotin was given in the diet at a level of 20 µg per 100 gm.

<sup>4</sup> Diets 11, 12, 13 and 15 differed from diets 7, 8, 9 and 10, respectively, only in that Labco casein instead of alcohol extracted casein was used and one drop weekly of vitamin D-enriched percomorph oil was substituted for the cod liver oil. Group 15 was given, by subcutaneous injection, 0.02 ml of Sharp and Dohme antipernicious anemia liver preparation instead of the crude liver extract, and the sucrose was increased to 74%. Diet 14 was the same as diet 13 except that the PGA was given intraperitoneally (10 µg per day).

dine supplement), and of the corresponding groups of experiments 2 and 3, gained weight at first but lost during the latter two or three weeks of the experiment, and a few died. For these animals the maximum weight attained was used in calculating the gain in weight. In experiment 1, two animals of group 2 died, but in both cases death occurred after the second blood count had been made.

As to gains in weight, table 2 shows that (a) the animals of group 1 (basal) grew very well, (b) those of group 2 (sulfasuxidine supplement) grew much less than any of the others, (c) the animals of groups 3, 4 and 5 (sulfasuxidine plus graded amounts of PGA) grew essentially the same as each other but definitely less than the controls, and (d) group 6 (sulfasuxidine plus liver extract) grew fully as well as the controls.

The weight data were analyzed statistically according to the method described in footnote 3 and the results are presented in table 3. Here are given the "t" values obtained from the statistical comparison of each group with every other group in an experiment. The difference in weight gain between groups 1 and 6 was not significant, but these two groups grew significantly more than any of the others; there was no real difference among groups 3, 4 and 5, but these all grew significantly more than the animals of group 2. As there was essentially no difference among groups 3, 4 and 5, it would appear that even the lowest level of PGA was sufficient to produce the maximum effect possible for this vitamin.

The white cell counts (table 2) were very nearly the same for the animals of the control group and of the three groups receiving PGA, while for group 6 they were slightly reduced and for group 2 they were about one-half those of the others. The neutrophils were still further reduced in the animals of group 2 and to a less extent in those of group 6. The percentage of this class of leukocytes for each of the 6 groups was 18, 9, 16, 20, 20 and 13, respectively. None of the animals showed any evidence of reduced hemoglobin.

*Experiment 2*

The probability existed that the large amount of sulfasuxidine may have partially destroyed some of the vitamins or prevented their complete absorption. Experiment 2 was designed to test this probability. There were 4 groups in this

TABLE 3

*"t" values for all possible comparisons within each experiment*

GROUP NO.	1	2	3	4	5	7 <sup>2</sup>	8	9	11	12	13	14
2	7.94 <sup>1</sup>											
3	3.29	6.77										
4	3.96	4.96	1.39									
5	5.42	4.94	0.81	0.42								
6	0.40	6.35	3.70	4.01	9.10							
8						11.0						
9						7.80	6.99					
10						0.17	7.28	4.85				
12									8.11			
13									2.48	9.67		
14									3.73	6.57	3.11	
15									0.071	20.9	4.19	3.84

<sup>1</sup> The "t" value for the 0.05% point for 6 degrees of freedom is 2.445 and for 5 degrees of freedom is 2.571.

<sup>2</sup> The different experiments are separated by double lines.

experiment (groups 7, 8, 9 and 10), corresponding to groups 1, 2, 4 and 6 of experiment 1. As it was shown above that the slower growth on the part of the animals receiving the sulfasuxidine and PGA was not due to insufficient PGA, only one level of this vitamin was included in the second experiment. The diets used for groups 7, 8, 9 and 10 differed from those of the corresponding groups of experiment 1 in that the



amount of thiamine chloride, riboflavin, pyridoxine, calcium pantothenate, i-inositol, nicotinic acid and 2-methyl-1,4-naphthoquinone was doubled, and biotin was included (20  $\mu$ g per 100 gm of diet).

The results of this experiment, shown in tables 2 and 3, are very much the same as those of the first experiment. Here again the animals of all other groups gained significantly more weight than the animals of group 8, while groups 7 and 10, which were nearly identical, gained significantly more than group 9.

One of the animals of group 8 died before the white cells were counted the second time, so the value for this group is the average of 6 observations. In general, the results obtained with the white cell counts were the same as in the first experiment. Two of the animals of group 8 were in a moribund condition when the counts were made and the neutrophil percentages were very high. This high count would indicate that these animals had an infection, but none was observed. Omitting these two animals, the average per cent of neutrophils for each of the 4 groups of experiment 2 was 14, 6, 18 and 11 respectively. Here again hemoglobin levels were essentially normal.

In the first experiment the animals receiving sulfasuxidine and liver extract had a slightly lower white cell count and a considerably lower neutrophil percentage than any of the groups receiving pteroylglutamic acid, and in the second experiment the total white cell count of the group receiving the liver extract was as high as the PGA group but the neutrophils were lower in the former than in the latter. Apparently the liver extract contained sufficient pteroylglutamic acid to produce good growth but not enough to give the same leukocyte picture as appeared in the animals receiving the synthetic compound.

### *Experiment 3*

The purpose of experiment 3 was to study the effect on growth of an antipernicious anemia liver concentrate in place of the crude liver extract, and to determine whether injecting

the PGA instead of giving it with the diet would allow the sulfasuxidine further to decrease growth. The basal or control diet was the high vitamin diet of experiment 2, except that vitamin-free casein<sup>5</sup> instead of the alcohol extracted casein was used and percomorph oil, one drop per week, was substituted for the cod liver oil. There were 5 groups of animals, the first three of which (11, 12 and 13) were fed diets the same as those fed groups 7, 8 and 9 of experiment 2; that is, basal, basal plus sulfasuxidine, and basal plus sulfasuxidine and 60  $\mu\text{g}$  % of PGA. Group 14 was given, in addition to the control diet, sulfasuxidine in the diet and 10  $\mu\text{g}$  of PGA per day by intraperitoneal injection. Group 15, in addition to the basal diet plus sulfasuxidine and 60  $\mu\text{g}$  % of PGA, was given subcutaneously 0.02 ml of antipernicious anemia liver concentrate<sup>6</sup> instead of the crude liver extract. White cell counts and hemoglobin determinations were not made on the animals of experiment 3.

The results of this experiment are also given in tables 2 and 3. Here again the average gains in weight of all other groups of animals were significantly more than those of group 12; the gains of group 11 just missed being significantly more than those of group 13, but the differences between 11 and 14 and 13 and 14, as well as between 15 and 14 and 15 and 13, were significant. None of the other pairs showed a real difference. It is to be noted that the average gain in weight for the group with the PGA in the diet was 8 gm more than for the group in which the PGA was given intraperitoneally. Although this difference is not great it was constant among the various pairs of animals, which accounts for the fairly high degree of significance. But the most striking result of this experiment was the excellent growth produced by the small amount of the antipernicious anemia preparation.

#### DISCUSSION

Nearly two decades ago Guha ('31) reported that milk contained a heat labile factor that increased the growth of young weanling rats when added to a diet of maize, wheat, "light

<sup>5</sup> Labco.

<sup>6</sup> Sharp and Dohme.

white" casein, powdered yeast,  $\text{CaCO}_3$  and  $\text{NaCl}$ . Sheep liver was less effective than milk. The following year Mapson ('32) observed that the addition of ox liver to a synthetic diet containing yeast produced larger rats than the same diet without the liver. White and Sayers ('42) obtained similar results using protein of pancreas. Hartman and Cary ('42) found that milk contained a growth promoting factor in rats when the B factors were supplied by yeast or yeast supplemented with all of the then known B vitamins, vitamin E, and vitamin K. Jaffé ('46) and Jaffé and Elvehjem ('47) grew rats on a diet of natural foods; here again growth was stimulated by liver or a laboratory prepared extract of fresh liver. The latter workers supplemented their diet of natural foods with synthetic B factors. They also obtained similar results with a synthetic diet. Sporn, Ruegamer and Elvehjem ('47) have demonstrated that rats born of females on a synthetic diet grow more slowly and have a higher mortality than the young born of dams on the same diet supplemented with liver.

More recent investigators who have made comparable observations are Bowland, Ensminger and Cunha ('48) and Zucker and Zucker ('48). The latter authors have emphasized the association of this growth factor with animal proteins.

Numerous reports have emphasized the need of the chick, the mouse, and certain bacteria for a nutrient found in several materials but especially in liver.

Finally, Rickes et al. ('48) succeeded in crystallizing a factor which they termed vitamin  $\text{B}_{12}$ . It has been shown that this crystalline compound is beneficial in Addisonian pernicious anemia (West, '48), and promotes the growth of *Lactobacillus lactis* (Shorb, '48), chicks (Ott et al., '48; Lillie et al., '48), rats (Emerson, Wurtz and Zanetti, '49), and pigs (Johnson and Neumann, '49; Hogan and Anderson, '49).

In most of the experiments referred to above in which purified casein was used as the protein, growth was poor unless the growth factor was added. In the experiments reported in the present paper, the growth of animals fed the purified protein was nearly as good as those receiving the sulfasuxidine

plus liver. The casein used in the first two experiments may not have been sufficiently purified, but in the last experiment vitamin-free casein was used.<sup>7</sup> Here again the average gains in weight of the animals receiving the liver extract and of the animals on the basal diet differed by only 3.5 gm. Hartman and Cary ('42) and Sporn et al. ('47) found this growth factor in milk, Mapson ('32), Sporn et al. ('47) and Bosshardt et al. ('49b) reported that it could be stored in the animal body, and Mapson showed that the factor could be carried through the mother's milk. Fresh milk is given *ad libitum* to our stock animals, and the young used in the experiments reported here were continued on the same regimen until they were put on experiment at 25 days of age. They undoubtedly had considerable stores of the necessary growth factor, which would explain the good growth of the animals on the basal diet.

It is impossible to conclude with finality that vitamin B<sub>12</sub> is the factor in liver which is responsible for the increased growth of our animals receiving the sulfa compound. However, since only 0.02 ml of the antipernicious anemia concentrate produced excellent growth in animals receiving PGA and sulfasuxidine, it is very possible that such is the case.

The mechanism by which succinylsulfathiazole increased the need for the growth factor is unknown at the present time. The most logical explanation is that intestinal bacteria form small amounts of this factor, and that the bacteria are inhibited by the sulfa drug from doing so. If that is the case, succinylsulfathiazole must have the power to produce bacterial stasis in the presence of pteroylglutamic acid.

As this manuscript was being prepared an abstract of a paper by Bosshardt et al. ('49a) appeared, reporting results with mice comparable to those presented here using rats.

#### SUMMARY

Young rats on a synthetic diet containing purified casein and all members of the vitamin B group except pteroylglutamic acid and vitamin B<sub>12</sub> grew well. The addition of succinylsulfa-

<sup>7</sup> See footnote 5, page 586.

thiazole produced the usual results, namely, failure of growth, leukocytopenia with the granulocytes affected more than the other cells, and finally death.

The inclusion of pteroylglutamic acid in the succinylsulfathiazole diet corrected the blood dyscrasia and prevented the death of the animals but failed to restore growth completely. Increasing the amount of pteroylglutamic acid did not change the picture.

Animals given succinylsulfathiazole plus a crude liver extract had normal blood and grew at a rapid rate.

Doubling the amount of 2-methyl-1,4-naphthoquinone and of the dietary B vitamins except choline did not increase the growth of rats given succinylsulfathiazole and pteroylglutamic acid.

The subcutaneous injection of 0.02 ml of a purified anti-pernicious anemia liver preparation in rats on the latter regimen produced very good growth.

The probable relation of this growth factor found in liver to vitamin B<sub>12</sub> is discussed.

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## OSBORNE AND MENDEL AWARD

Nominations are invited for the Osborne and Mendel Award of \$1000.00 established by the Nutrition Foundation, Inc. for the recognition of outstanding accomplishments in the general field of exploratory research in the science of nutrition. It shall be given to the investigator who, in the opinion of a Jury of Award, has made the most significant published contribution in the year preceding the annual meeting of the Institute, or who has published a series of contemporary papers of outstanding significance.

The Award will be presented at the annual meeting of the American Institute of Nutrition.

The recipient will be chosen by a Jury of Award of the American Institute of Nutrition. As a general policy, the Award will be made to one person. If, in the judgment of the Jury of Award, an injustice would otherwise be done, it may be divided among two or more persons. Normally preference will be given to research workers in the United States and Canada, but investigators in other countries, especially those sojourning in the United States or Canada for a period of time, are not excluded from consideration. Membership in the Institute of Nutrition is not a requirement for eligibility and there is no limitation as to age.

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*University of Illinois*  
*Urbana, Illinois*

CHAIRMAN, NOMINATING COMMITTEE

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The formal presentation will be made at the annual meeting of the Institute in the spring of 1950. To be considered for the award, nominations must be in the hands of the Chairman of the Nominating Committee by January 15, 1950. The nominations should be accompanied by such data relative to the nominee and his research as will facilitate consideration for the award.

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*Cornell University, Ithaca, New York*

CHAIRMAN, NOMINATING COMMITTEE



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The Award will be given to the laboratory or clinical research worker in the United States or Canada who, in the opinion of the judges, has published during the previous calendar year, January 1 to December 31, the most meritorious scientific report dealing with the field of the 'B-complex' vitamins. While the award will be given primarily for publication of specific papers, the judges are given considerable latitude in the exercise of their function. If in their judgment circumstances and justice so dictate, it may be recommended that the award be made to a worker for valuable contributions over an extended period but not necessarily representative of a given year. Membership in the American Institute of Nutrition is not a requisite of eligibility for the award.

To be considered by the Committee of Judges, nominations for this award for work published in 1949 must be in the hands of the Chairman of the Nominating Committee by January 15, 1950. The nominations should be accompanied by such data relative to the nominee and his research as will facilitate the task of the Committee of Judges in its consideration of the nomination.

W. H. SEBRELL, JR.  
*Experimental Biology and  
Medicine Institute  
National Institutes of Health  
Bethesda, Maryland*

CHAIRMAN, NOMINATING COMMITTEE



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